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Skeletal Growth in Pyridoxine Deficient Mice.*

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The present investigation deals with the histological changes in the long bones associated with the arrest of skeletal growth in pyridoxine deficient mice.

Material and Methods. Twenty-eight male and female mice of strain C57 black, 28 days old, were used. Fourteen animals were placed on a synthetic diet complete except for the lack of pyridoxine (Group I). Fourteen animals were placed on a synthetic diet complete except for pyridoxine but containing 54% casein (Group II). Animals of corresponding strain and age, fed a stock diet (Purina Laboratory Chow), used in previous experiments served as normal controls. The rations were composed as follows:

	Group I (Pyridoxine free)	Group II (Pyridoxine free, high protein, low sucrose)
Sucrose	68 g	30 g
Vit. test casein	18 "	54 "
Vegetable oil	10 "	10 "
U.S.P. salt mix. No. 12	4 "	4 "

* Aided by a grant of the Committee on Scientific Research of the American Medical Association and the U. S. Public Health Service.

Cod liver oil	1 cc	1 cc
Riboflavin	0.5 mg	0.5 mg
Thiamin	0.2 "	0.2 "
Nicotinic acid	1.0 "	1.0 "
Choline HCl	1.0 "	1.0 "
Inositol	100.0 "	100.0 "
Ca pantothenate	10.0 "	10.0 "
Biotin	25 μg	25 μg

The animals were kept on the deficient diets for 1, 2, 4 or 8 weeks. Of those fed the deficient diets for 4 weeks, 8 were refed the stock diet (Purina Laboratory Chow) for 1, 3, 5, 10 or 14 days. Paraffin sections of the kneejoint were prepared and stained with hematoxylin and eosin.

Group I. After 1 week's deficiency, the epiphyseal discs at the upper end of the tibia were narrowed from the normal 300 to 170 microns. The cartilage cells were decreased in size, and instead of the usual 4 hypertrophic and 10 columnar cells there were 3 hypertrophic and 7 columnar cells in the individual cartilage cell row. The intercellular matrix was somewhat increased. The metaphysis was vascular, and the trabeculae were covered by numerous osteoblasts. The cortex measured 85 microns in diameter which is about normal. After 2 weeks, the epiphyseal discs were 150 microns wide and contained 2 or 3 cells of hypertrophic and 6 of columnar type. Both cell types were markedly reduced in size. The metaphyseal spicules were shortened and linked with one another. The osteoblasts were more oblong than ordinarily. Severe changes were noted after 4 weeks' deficiency: The epiphyseal discs measured 90 microns in width, and the cartilage cells had undergone additional shrinkage; the cartilaginous matrix was more abundant and denser. The few trabeculae present were short, and here and there, interlaced. The osteoblasts in the metaphysis and along the endosteum were spindle-shaped and markedly decreased in number. Osteoclasts were scarce. The cortex of the shaft was thinned out, and particularly at the junction of the epiphyseal disc and the shaft ("Umbauzone"), there was a conspicuous decline of activity: Little bone was present, osteoblasts were scanty and small, and the precartilage usually arising from the perichondrium failed to develop. The articular cartilage showed corresponding atrophy. After 8 weeks, the epiphyseal discs as well as the peripheral areas of remodeling presented a picture of complete inactivity. The former measured 50 microns in width. The individual cartilage cell row contained 4 to 6 very small columnar and 2 shrunken cells of hypertrophic type. Hyalinization of the matrix was widespread, and in the distal third of the epiphyseal disc dense deposits of calcium were noted. Vascular erosion of the cartilage had ceased, and bony trabeculae were lacking. A very thin often membrane-like bony lamella delimited the cartilage from the marrow. The few fragments of osseous spicules present in the distal metaphysis were covered by a discontinuous layer of spindle-shaped cells. The cortex of the shaft measured in some places as little as 20 microns in diameter and was considerably thinner than the fibrous periosteum. The cartilage of the joint was atrophic.

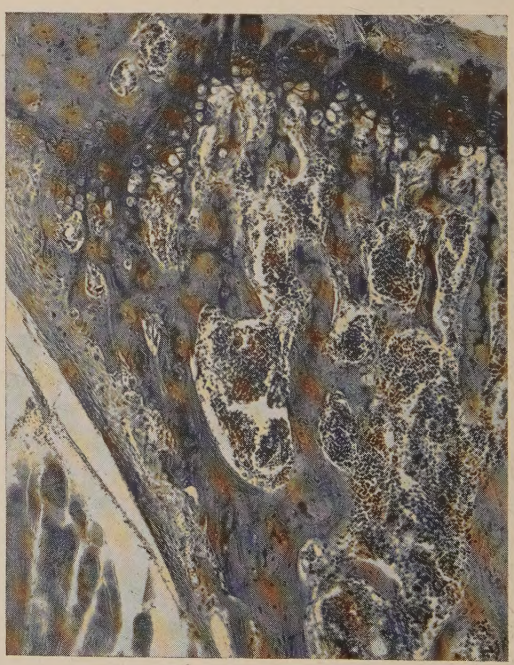

FIG. 1.

Section through the upper end of the tibia of a female mouse of strain C57, 12 weeks old, fed the stock diet. The growth zone is hyalinized and calcified. The hypertrophic cartilage cells are distinct. The bony spicules are long and thick. The shaft contains abundant bone. Medium high magnification.

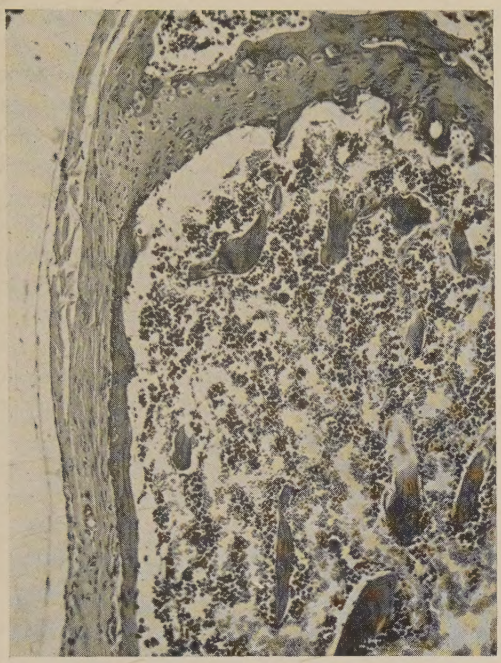

FIG. 2.
Section through the upper end of the tibia of a female mouse of strain C57, 12 weeks old, kept on a pyridoxine-deficient diet high in protein for 8 weeks. There are no hypertrophic cartilage cells, and bony trabeculae are rudimentary. A thin osseous lamella delimits the epiphyseal cartilage from the bone marrow. The shaft is markedly thinned out. Same magnification as No. 1.

There were no definite sex differences in the susceptibility to pyridoxine deficiency.

The early effects of refeeding were observed after 3 days: The epiphyseal discs at the upper end of the tibia were about 160 microns wide as compared with 90 microns after 4 weeks of deficiency. The hypertrophic cartilage cells were larger and their number had increased from 1 to 3 cells. The columnar cells likewise appeared larger and mitotic proliferation was resumed. The new formation of cartilage from the perichondrium at the periphery of the epiphyseal discs was particularly conspicuous. After 5 days of refeeding, the epiphyseal disc was 200 microns wide and thus only one-third narrower than usual. Columnar and hypertrophic cartilage cells had further increased in size, and they were present in almost normal numbers (3:9 as compared with a normal of 4:10). Osteoblasts were numerous, although they were still more oblong than ordinarily. The cortex had become thicker and measured 90 microns. During the second week of refeeding, the growth processes were restored to almost normal, and the epiphyseal discs were even somewhat wider than in normally fed animals of corresponding age. Mitoses were not infrequent in the proliferating cartilage cells. However, there were still some increase of the matrix and slight atrophy of the cartilage cells. Replacement of the hypertrophic cartilage cells by bone proceeded at a normal rate; osteoblasts were abundant and large. The cortex and articular cartilage were of usual structure.

Group II. During the first 2 weeks, the effect of the high protein on the pyridoxine deficiency was not definite. However, after 4 weeks a distinctly unfavorable influence of the addition of casein became noticeable. The growth zones at the upper end of the tibia measured 60 microns in width as com-

FIG. 3.
Section through the upper end of the tibia of a female mouse of strain C57, 9½ weeks old, kept on a pyridoxine-deficient diet for 4 weeks, and refed the stock diet for 10 days. The epiphyseal disk is wide, cellular and has a youthful appearance. Growth, development, and osseous replacement of cartilage are in progress. Much bone is present. Same magnification as No. 1.

FIG. 4.
High power photograph of a bony trabecula of the same animal as in Fig. 1. The spicule is thick and is covered by a continuous layer of large osteoblasts.

pared with 90 microns in mice kept on the deficient diet with regular protein content. The cells were smaller and fewer than in the latter group, and the matrix was more hyalinized. These differences were accentuated after 8 weeks and manifested themselves chiefly in the absence of hypertrophic cartilage cells and in even more advanced hyalinization of the matrix.

Resumption of growth after refeeding was slower in the group receiving the high protein diet. A noteworthy enlargement of the epiphyseal discs was not seen until after 5 days of realimentation. Even after 10 days, the cartilage cells were still smaller than ordinarily, although the individual cartilage cell row contained the usual number of cells. Mitotic proliferation of the cartilage cells was less prominent indicating a comparatively slow rate of growth. Moreover, plugs of degenerated cartilage and hyalinization of the ground substance were still noticeable. Osteoblasts were rather scanty, and they had not yet completely regained their epithelioid shape.

Summary and Conclusions. In pyridoxine deficient young mice, growth of cartilage and formation of bone were inhibited and finally ceased. Upon refeeding the complete diet, recovery was noticeable after 3 days and was still in progress after 14 days. The growth processes were quickly restored with mitoses present in the germinal layer of the cartilage as well as in the osteoblasts.

The effects of pyridoxine deficiency on cartilage resemble those seen in pantothenic acid deficient mice,[1] whereas the disturbance of ossification was not unlike that observed in riboflavin deficiency.[2] The specificity of the skeletal changes may thus be questioned.

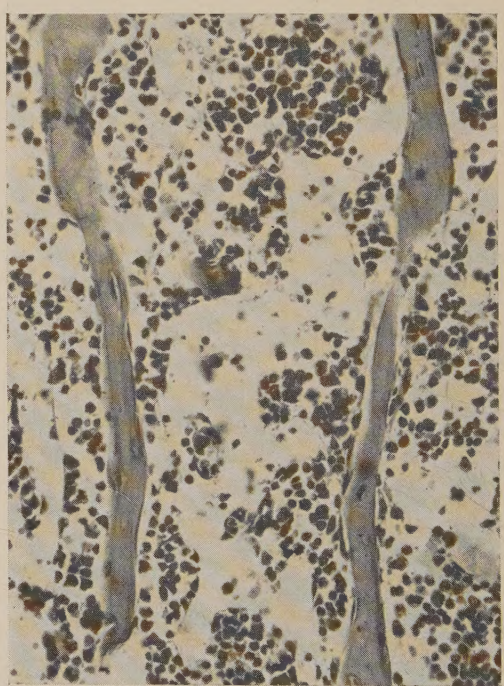

FIG. 5.
High power photograph of 2 bony trabeculae of a female mouse of strain C57, 12 weeks of age, and fed a pyridoxine-deficient diet for 8 weeks. The spicules are thin and covered only here and there by spindle-shaped cells. Osteoblasts are not seen. The bone marrow is atrophic. Same magnification as No. 4.

1 Levy, B. M., and Silberberg, M., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 380.

2 Levy, B. M., and Silberberg, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 355.

In young rats fed a pyridoxine deficient diet,[3] the changes in the growth zones were comparable to those of underfed young guinea pigs,[4] kept on a quantitatively restricted but adequately balanced diet. To some extent this is true for the cartilage of mice also. However, the cartilage cells as well as the osteoblasts responded more unfavorably to the pyridoxine deficiency than to general underfeeding, and the formation of bone was more reduced than in any of our previous experiments. This difference might indicate a specific effect of the pyridoxine deficiency, but it may be a manifestation of the more marked sensitivity of the mouse to the deficiency as compared to that of the rat.[5,6]

Feeding a high protein diet accentuated the effects of pyridoxine deficiency inasmuch as growth of cartilage and bone was more inhibited than in pyridoxine deficient mice receiving a diet with regular casein content. These findings are in agreement with previous observations on the greater requirements for pyridoxine in animals fed high protein diets.

3 Antopol, W., and Unna, K., *Arch. Path.*, 1942, **33**, 241.

4 Silberberg, M., and Silberberg, R., *Arch. Path.*, 1940, **30**, 675.

5 Cerecedo, L. R., and Foy, J. R., *Arch. Biochem.*, 1944, **5**, 207.

6 Miller, E. C., and Baumann, C. A., *J. Biol. Chem.*, 1945, **157**, 551.

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Experimental Fort Bragg Fever (Pretibial Fever) in Chimpanzees.*

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The chimpanzee (*Pan Satyrus*) has been used extensively in experimental work on poliomyelitis[1,2] and to a lesser degree in work on the common cold,[3] but the use of this animal as an experimental host for the reproduction of human infectious disease is a field deserving further exploration. Particularly would this seem to apply to the virus field and to diseases which have readily discernible cutaneous manifestations. We have recently made attempts to induce in these animals 3 human virus diseases: sandfly (Phlebotomus or pappataci) fever, dengue fever, and Fort Bragg (pretibial) fever. This paper will be concerned only with a description of our experiments with the latter disease. Results with the other two diseases will be described in another paper.[4]

Fort Bragg fever was first described by Daniels and Grennan,[5] under the term *pretibial fever*. It is a disease characterized by an acute fever of approximately 5 days' duration, frontal and postorbital aching, splenomegaly, and often with a patchy erythematous rash on the pretibial regions.† Respiratory manifestations are minimal and not persistent. Cases of this disease have appeared in groups during the summer months of 1942, 1943, and 1944 on the military reservation at Fort Bragg, N. C. The manner in which it spreads among humans has not been determined.

* This investigation was conducted by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

1 Bodian, D., and Howe, H. A., *J. Exp. Med.*, 1947, **81**, 255.

2 Melnick, J. L., and Horstmann, D. M., *J. Exp. Med.*, 1947, **85**, 287.

3 Dochez, A. R., Shibley, G. S., and Mills, K. C., *J. Exp. Med.*, 1930, **52**, 701.

4 Paul, J. R. Melnick, J. L., and Sabin, A. B., to be published.

5 Daniels, W. B., and Grennan, H. A., *J.A.M.A.*, 1943, **122**, 361.

† The rash on the shins was such a prevalent feature that Daniels and Grennan[5] have designated this disease as *pretibial fever*.

This disease has been studied by Tatlock[6] who recovered an infectious filtrable agent from an active case. This agent produced a prostrating fatal infection in hamsters and fever in guinea pigs and rabbits. The agent was also maintained for 23 serial passages in embryonated eggs, following which the virus was inoculated into 14 human volunteers. It was possible to induce the clinical picture of Fort Bragg fever including the characteristic skin lesions in some of these subjects, while the majority exhibited only fever for 1 to 3 days. The virus appeared to be unrelated in its properties or immunologically, to the agents of lymphocytic choriomeningitis, Q fever, Rocky Mountain spotted fever, sandfly fever, and dengue fever.

Virus. The strain was received from the Virus and Rickettsial Laboratory of the Army Medical School through the kindness of Dr. Hugh J. Tatlock and Dr. Joseph E. Smadel. Because of difficulties of preserving the infectiousness of the virus on storage, even in sealed glass ampoules at –70°C[6], the virus was best handled by maintaining it in serial passage in hamsters. Only an occasional sample of virus survived storage on dry-ice, a fact which on more than one occasion almost resulted in our losing the virus.

Most consistent results were obtained when young hamsters (3 to 4 weeks of age) were used. Virus was inoculated intracerebrally (0.04 cc) and/or intraperitoneally (0.5 cc), generally as a 10% suspension of brain. Following an incubation period generally from 7 to 18 days, animals were sacrificed and brains harvested for virus on the first day of illness. Prostration and death usually developed within 24 hours of the first signs of disease.

Chimpanzees. Nine young animals were used in these experiments.‡ All of them had been used during the previous one to 3 years for work in poliomyelitis and several of them had developed the non-paralytic form of poliomyelitis. This did not seem to be a contraindication to their use with Fort Bragg fever virus. It should be added, however, that none of these chimpanzees were harboring the virus of poliomyelitis in their intestines at the time of using them for these experiments with Fort Bragg fever virus. All of these animals were under 5 years of age but only 4 of them could be described as being tractable, in other words as being handled easily enough to allow the taking of daily rectal temperatures. All animals, however, were examined daily for signs of illness for a period of 5 to 6 weeks. Animals were bled before inoculation, at the time of fever, and at various intervals after inoculation of virus. Virus was inoculated as indicated, intra- and subcutaneously and sometimes intramuscularly.

Neutralization Tests. These were carried out in hamsters. Suspensions of freshly harvested brains from sick hamsters were made up into varying dilutions ($2x10^{-1}$ to $2x10^{-5}$ concentration of brain). These were mixed with equal amounts of undiluted serum and incubated for one hour at room temperature. The virus-serum mixtures were inoculated into groups of 3 young hamsters either intracerebrally (0.04 cc) or intraperitoneally (0.5 cc) as indicated. Some sera were tested by both the intracerebral and the intraperitoneal routes, in view of the discrepancy which is sometimes found between these two routes in certain virus neutralization tests.[7] In general the intraperitoneal route proved satisfactory,[6] and was most often used. Hamsters were observed for a period of 21 days for signs of illness and death.

Exp. I. Preliminary experiments carried out with 4 chimpanzees in November, 1946 were equivocal. In these animals temperature readings were taken on 2, and fever was recorded in one. But there were no hamster controls to indicate that the sample of virus used was active. Furthermore, the virus was not isolated from the blood of the chimpanzees during the febrile period and no neutralizing antibodies were demonstrable in the convalescent samples (see Table I). The experiments were repeated in March, 1947.

Exp. II. 10 March 1947. Chimpanzee Rosebud and 2 rhesus monkeys were each

[6] Tatlock, H. J., *J. Clin. Invest.*, 1947, **26**, 287.

‡ We are indebted to Dr. Karl S. Lashley of the Yerkes Laboratories of Primate Biology, Orange Park, Florida, for the loan of 6 of these animals.

[7] Casals, J., and Olitsky, P. K., *J. Exp. Med.*, 1945, **82**, 431.

TABLE I.
Lack of Development of Neutralizing Antibodies in Chimpanzees Pinta, Webb, and Jent. (Inoculated with Virus Inactivated by Freezing and Thawing.)

Serum of chimpanzee	Date, 1946	Inoculation route in hamsters	Mortality at indicated concentration of infectious hamster brain					Result of antibody test
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Pinta	12 Nov.	IC		3/3	0/3	0/3	0/3	—
”	11 Dec.	IC	3/3	3/3	1/3			—
”	12 Nov.	IP		3/3	3/3	0/3	0/3	—
”	11 Dec.	IP	4/4	3/4	3/4			
Webb	12 Nov.	IC		3/3	3/3	0/3	0/3	—
”	11 Dec.	IC	3/3	3/3	2/3			—
”	12 Nov.	IP		3/3	3/3	2/3	0/3	—
”	11 Dec.	IP	3/3	3/3	3/3			—
Jent	12 Nov.	IC		3/3	1/3	1/3	0/3	—
”	11 Dec.	IC	3/3	3/3	2/3			—
”	12 Nov.	IP		3/3	3/3	1/3	0/3	—
”	11 Dec.	IP	3/3	3/3	3/3			—

TABLE II.
Development of Neutralizing Antibodies to Fort Bragg Fever Virus in Chimpanzees.

Serum of chimpanzee	Date 1947	Material inoculated	Inoculation route in hamsters	Mortality at indicated concentration of infectious hamster brain					Result of antibody test
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Rosebud	10 March	Active virus	IP		2/2	3/3	3/3		—
”	23 June	” ”	IP	0/3	0/3	0/3	0/3		+ 1000
”	31 July		IP	0/3	0/3	0/3			+ 1000
Mary-Lou	20 March	Rosebud's febrile blood	IP		3/3	3/3	2/3		—
”	30 April		IP	0/3	0/3	0/3			+ 1000
”	23 June	Active virus	IP	1/3	0/3	0/3	0/3		+ 100
Hickory	29 April	Inactive ”							
”	23 June	Active ”	IP		3/3	1/2	0/3		—
”	31 July		IP	0/3	0/3	0/3			+ 100
Catawba	29 April	Inactive ”	IP		2/2	2/2	1/3		—
”	23 June	Active ”	IP		3/3	2/2	3/3		—
”	31 July		IP	0/3	0/2	0/3			+ 1000
Becky	23 June	” ”	IP		3/3	3/3	2/3	0/3	—
”	31 July	” ”	IP	0/3	0/3	0/3	0/3		+ 1000
”	23 June	” ”	IC		3/3	3/3	1/3	0/3	—
”	31 July	” ”	IC	2/3	0/3	0/3	0/3		+ 100

+ 1000 = Undiluted serum neutralized 1000 lethal doses of virus.

inoculated intra- and subcutaneously (2 cc in several piqures) and intramuscularly (3 cc) and 2 other rhesus monkeys intracerebrally (0.6 cc) with a 20% suspension of infectious hamster brains. These brains were harvested from 5 hamsters ill with the disease on that day, and the suspension was inoculated soon after it was prepared. As controls, 5 new hamsters were inoculated intracerebrally with the virus suspension.

5 Hamsters—10 March. Inoculated with virus.
20 March. One dead, 4 sick with tremors, ataxia, and prostration. Sacrificed for virus harvest.
4 Rhesus Monkeys—10 March. Inoculated with virus.
11-30 March. Normal behavior and temperature.
Chimpanzee Rosebud—10 March. Bled for serological antibody test (Test negative; see Table II). Inoculated with virus.
11-19 March. Normal, temp. between 98° and 99.7°. White blood count between 16,000 and

22,000.

20 March. Temp. 101.7°. White blood count 35,500. Twenty-five cc of blood taken, citrated, and 10 cc inoculated within 5 minutes into Chimpanzee Mary-Lou (7 cc intramuscularly and 3 cc intra- and subcutaneously in 15 piqures). Remainder of citrated blood *frozen* and later after thawing, tested in 6 hamsters with negative results.

22-23 March. One lesion on right shin and one on left forearm, as raised red areas about 1 cm in diameter. Temp. 101°. White blood count 12,800.

24 March. Normal. Temperature 99°. White blood count 17,800.

29-30 March. Normal. Temp. 99.1° to 99.8°.

23 June. Bled for antibody test (Test positive; see Table II).

Chimpanzee Mary Lou—20 March. Bled for serological antibody test (Test negative; see Table II). Inoculated with Rosebud's citrated blood.

21 March-30 April. Normal. Temp. not taken.

30 April. Bled for antibody test (Test positive; see Table II).

Exp. III. 29 April 1947. Chimpanzees Catawba and Hickory were each inoculated intra- and subcutaneously (2 cc) and intramuscularly (1 cc) with 20% suspension of hamster brains. The suspension was made on the day of inoculation from 4 infected hamster brains which had been stored on dry-ice for 5 weeks. As controls 10 new hamsters were inoculated intracerebrally.

10 Hamsters—29 April. Inoculated.

30 April-19 May. Remained well.

Chimpanzee Hickory. 29 April. Bled for antibody test. Inoculated.

30 April-19 May. Normal temp. 98.4° to 99.9°.

23 June. Bled for antibody test (Test negative; see Table II).

Chimpanzee Catawba—29 April. Bled for antibody test. Inoculated.

30 April-19 May. Normal. Temp. not taken.

23 June. Bled for antibody test (Test negative; see Table II).

Exp. IV. 23 June 1947. Five chimpanzees were inoculated with 10% brain suspension freshly prepared from 2 hamsters acutely ill with Fort Bragg fever. Two of these chimpanzees (Rosebud and Mary-Lou) had previously been inoculated with active virus and 2 (Hickory and Catawba) with virus inactivated by storage on dry ice. The fifth animal (Becky) had not been used in Fort Bragg fever work before the present experiment. All 5 chimpanzees were each inoculated intra- and subcutaneously with 2 cc of the virus suspension in 6 to 8 piqures. As controls 9 new hamsters were inoculated intracerebrally with the same suspension.

9 Hamsters—23 June. Inoculated.

3 July. Two animals hyperirritable, tremulous, and ataxic (sacrificed for virus).

4 July. Three animals ataxic progressing to prostration (sacrificed for virus).

5 July. One dead.

6 July. One dead. One sick as those above.

7 July. Two dead.

Chimpanzee Rosebud (previously inoculated with active virus)—23 June. Bled for antibody test (Test positive; see Table II). Inoculated with virus.

24 June-31 July. Normal. Temp. 97° to 99.6°.

31 July. Bled for antibody test (Test positive; see Table II).

Chimpanzee Mary-Lou (previously inoculated with Rosebud's febrile blood)—23 June. Bled for antibody test (Test positive; see Table II). Inoculated with virus.

24 June-31 July. Normal. Temp. not taken.

Chimpanzee Hickory (previously inoculated with inactived virus)—23 June. Bled for antibody test (Test negative; see Table II). Inoculated with virus.

24 June-9 July. Normal. Temp. 98° to 99.6°.

10-12 July. Temp. elevated 100.8° to 101.2°.

14-31 July. Normal. Temp. 97.6° to 99°.

31 July. Bled for antibody test (Test positive; see Table II).

Chimpanzee Catawba (previously inoculated with inactived virus)—23 June. Bled for antibody test (Test negative; see Table II). Inoculated with virus.

24 June-31 July. Normal. Temp. not taken.

31 July. Bled for antibody test (Test positive; see Table II).

Chimpanzee Becky (new animal)—23 June. Bled for antibody test (Test negative; see Table II). Inoculated with virus.

24 June-25 July. Normal. Temp. 97.1° to 98.3°.

3 July. Temp. 100.5°.

4 July. Temp. 100°. Blood taken and 15 cc defibrinated. Inoculated at once by the intracerebral and intraperitoneal routes into 4 young hamsters. All 4 succumbed to the

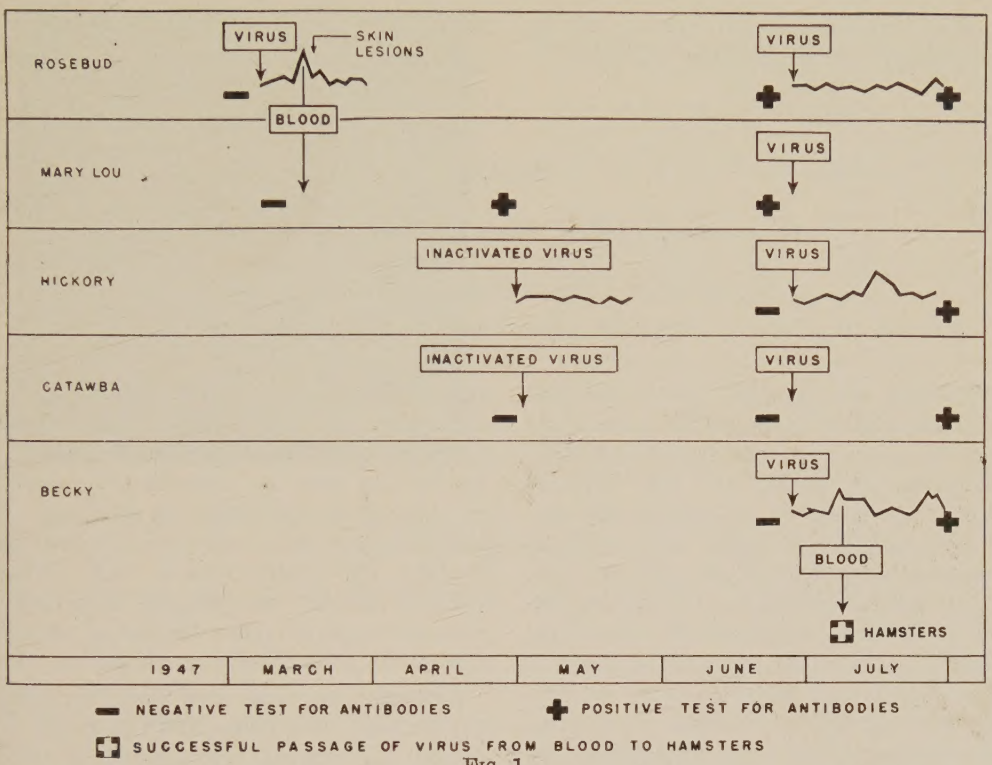

FIG. 1.

Fort Bragg fever in the chimpanzee. The temperature curves of 3 animals are presented together with the results of antibody tests on all 5 animals.

disease (8- to 10-day incubation period) and their brains were shown to contain the virus by another successful subtransfer into new hamsters.

9 July. Temp. back to 99°.

31 July. Bled for antibody test (Test positive; see Table II).

Discussion. The results of this study which are graphically presented in Fig. 1, indicate that chimpanzees are susceptible to the virus of Fort Bragg fever when administered intra- and subcutaneously. The induced disease is similar in many respects to the experimental disease in human beings which has been reported by Tatlock.[6]

A short febrile period developed after 10 to 17 days in the 3 chimpanzees who were tractable enough to have their daily temperatures taken. In the 2 animals in whom the presence of viremia was sought during the febrile period, virus was recovered, in one animal by transfer to another chimpanzee, in the second by transfer to hamsters.

The development of neutralizing antibodies to the virus is further evidence that infection had taken place in these animals. When inactivated virus (inactivated by freezing and thawing whole infected hamster brains) was inoculated into 5 chimpanzees (Hickory, Catawba, Pinta, Webb, and Jent), neutralizing antibodies did not appear in the serum. However, when 2 of these animals (Hickory and Catawba) were later challenged with active virus, both responded by developing antibodies to the virus.

Of considerable importance is the fact that a rash did appear in the appropriate location, the shin and forearm of an inoculated animal. This was not a very constant phenomenon as in the human disease, for out of the 14 subjects inoculated by Tatlock 13 responded with fever and of these, only 5 developed a rash.

Summary. Inoculation of Fort Bragg fever virus into chimpanzees resulted in a mild infection. This appeared to be characterized

by a short febrile period with viremia and by the appearance of neutralizing antibodies in the serum a few weeks after the infection.

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Conversion of Red Muscle to Pale Muscle.*

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It has long been taught that the function of myohemoglobin in red muscle is its function as an oxygen reservoir for the sustained contractions so typical of this kind of muscle.[1] Variations in myohemoglobin content were noted by Whipple[2] who correlated the content of this pigment with the age, state of health, and amount of activity engaged in by dogs. McClintock, Hines, and Jordan[3] observed that increased activity of muscle would lead to an increase in myohemoglobin content. In order to test the validity of the concept that myoglobin content and type of activity are correlated, the experiments described below were carried out.

Methods. Large, mature rabbits were selected for these experiments. Six such animals were used and each was anesthetized with nembutal. Under aseptic conditions, the tendon of the right soleus muscle was severed and the central end of this tendon sutured to the peripheral end of the severed tendon of the synergistic pale tibialis posterior muscle. By causing the red soleus to utilize the insertion of the pale tibialis muscle, it was hoped to convert the soleus to a pale muscle. The ankle was fixed with an orthoplast bandage in such a way that the foot was kept partially flexed for a period of 2 weeks at the end of which time the bandage was removed. The animals were then sacrificed after a period of 6 months. At sacrifice, the animal was placed under nembutal anesthesia and the normal tibialis and soleus of the left leg and the transplanted soleus of the right leg were arranged for recording. All tendon sutures were found to have been successful. The contractions of each of these extensor muscles were elicited as a part of a crossed extensor reflex elicited by stimulation of the contralateral sciatic nerve.

After the recordings were made, the animal was perfused with saline, and the muscle was excised and weighed. Hemoglobin of muscle was determined by the acid hematin method according to Whipple[4] and iron determinations were simultaneously made by the method of Wong.[5]

Results A comparison of reflexly induced tetanic contractions is shown in Fig. 1. This typical result illustrates the fact that excitation with stimuli of equal strength (10 volts), equal frequency (60 c.p.s.), and equal duration (1 sec.) result in a prolonged and maximal degree of contraction, marked by considerable after-discharge in the normal soleus and a very short, lesser degree of tetanus with a conspicuous lack of after-discharge in the case of the normal tibialis posterior. The transplanted soleus, on the other hand, exhibits a reflexly induced contraction which is remarkably similar to the contraction of the pale tibialis muscle. There remain, however, some remnants of red muscle contraction characteristics as evidenced by the greater extent of contraction than with the pale muscle and by the existence of a

* Aided by a grant from the David Trautman Schwartz Research Fund of the Tulane School of Medicine.

1 Millikan, G., *Physiol. Ref.*, 1939, **19**, 509.

2 Whipple, G. H., *Physiol.*, 1926, **76**.

3 McClintock, J. T., Hines, H. M., and Jordan, D. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 139.

4 Whipple, G. H., *Physiol.*, 1926, **76**, 693.

5 Wong, S. Y., *Biol. Chem.*, 1928, **77**, 409.

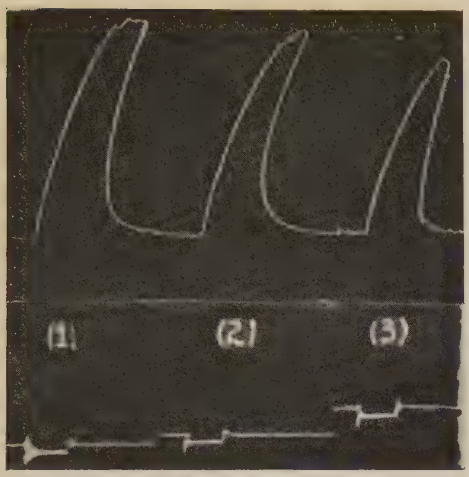

FIG. 1.
Kymograms showing the effect of stimulation of the contra-lateral sciatic nerve in causing reflex contraction of (1) the normal (left) soleus, (2) the transplanted (right) soleus, (3) the normal (left) tibialis posterior.

minimal degree of after-discharge. It is apparent, nevertheless, that the characteristic of the contraction is more nearly that of pale muscle than of red.

In Table I are compared the analyses for iron and myohemoglobin in normal soleus, transplanted soleus, and normal tibialis posterior muscles. It is evident from these results that the alteration in function of the red muscle has resulted in an iron and myohemoglobin content very similar to that of normal pale muscle and far below that of normal red muscle.

TABLE I.
Comparison of Myohemoglobin and Iron Contents in Normal Red, Transplanted Red, and Normal White Muscles.
All muscle weights corrected to 5 g. All values represent averages in mg per 5 g muscle of the number of determinations indicated in parentheses.

Muscle	Iron content	Myohemoglobin content
Tibialis posterior (2)	32.50	10.00
Transplanted soleus (3)	34.00	10.29
Normal soleus (3)	53.49	15.74

Summary. Conversion of red muscle to pale muscle can be accomplished by changing the position of the tendon of insertion to that of a white muscle. In thus assuming the function of a pale synergistic muscle, the red muscle in turn assumes the properties of pale muscle as evidenced by similar types of reflexly induced contractions and by similar myohemoglobin and iron contents. Acknowledgment is due Mrs. G. W. Wyatt for aid in the chemical determinations.

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Sulfhydryl and Disulfide Content of Normal and Arsenic-Resistant Trypanosomes.*

STEWART C. HARVEY.† (Introduced by E. M. K. Geiling.)

The sulfhydryl group has been regarded as a more or less specific receptor for tervalent arsenicals since it was postulated by Voegtlin[1] that arsenicals are bound through the formation of dithioarsenite complexes. It was further suggested by him[2] that differences in the equilibrium position of the glutathione system or in the absolute quantity of glutathione in sub-strains of a given strain of trypanosomes were responsible for the phenomenon of arsenic-resistance, the resistant strain presumably having a greater sulfhydryl reserve with which to detoxify the arsenical. Since then, the concept of the arsenic receptor has been broadened to include the fixed sulfhydryl groups of proteins,[3,4] the importance of which has been emphasized by the work of Barron

* Supported by a grant-in-aid from the U. S. Public Health Service.

† John Jacob Abel Fellow in Pharmacology.

1 Voegtlin, C., Dyer, H. A., and Leonard, C. S., *U. S. Pub. Health Rep.*, 1923, **38**, 1882.

2 Vogetlin, C., Dyer, H. A., and Miller, D. W., *J. Pharm. and Exp. Therap.*, 1924, **23**, 55.

3 Voegtlin, C., and Rosenthal, S. M., *J. Pharm. and Exp. Therap.*, 1930, **39**, 347.

and Singer.[5] The presence of the sulfhydryl group has been demonstrated in *T. equiperdum* by Voegtlin,[1] but no quantitative estimations have hitherto been reported. Reiner *et al.*[6,7] report that under comparable conditions of exposure, normal and resistant strains of *T. equiperdum* bind essentially the same amount of arsenic, irrespective of the drug used, which offers indirect evidence that the sulfhydryl group is not involved in the phenomenon of arsenic-resistance. In a more direct attempt to elucidate the role of the sulfhydryl group in arsenic resistance, a quantitative study of the sulfhydryl and disulfide contents of normal and oxophenarsine-resistant strains of *T. equiperdum* and *T. hippicum* was made.

Materials and Methods. The normal strain of *T. equiperdum* was one obtained from the laboratory of Dr. A. L. Tatum, and the oxophenarsine-resistant strain was a substrain produced in this laboratory.[8] The normal strain of *T. hippicum* was obtained from Dr. M. H. Soule and the resistant strain from Dr. S. H. Sandground. The trypanosomes were propagated in a Sprague-Dawley strain of albino rats.

The protein samples were prepared in the following manner: When the number of trypanosomes in the blood of the rat rose above 500,000 per cmm, the animal was decapitated and exsanguinated into a beaker which held 2 cc of heparinized Ringer-Locke solution (U.S.P.XIII) containing 0.75% glucose and 0.05% sodium bicarbonate. The volume of blood obtained in this way was usually 7-13 cc. The blood was centrifuged 8 minutes, the trypanosome layer was pipetted into 10 cc of the above Ringer-Locke solution, and then recentrifuged. This process was repeated until the trypanosomes were free

TABLE I. Sulfhydryl and Disulfide Content of Two Strains of *T. equiperdum* Expressed as the Cysteine Equivalents.

Normal strain				Arsenic-resistant strain			
Sulfhydryl mg/g	Combined mg/g	Disulfide mg/g		Sulfhydryl mg/g	Combined mg/g	Disulfide mg/g	
Mean	Standard error of mean	Difference	Standard error of diff.	Mean	Standard error of mean	Difference	Standard error of diff.
0.32	±0.05 (17 analyses)	6.00	±0.04 (28 analyses)	5.68	±0.06	0.40	±0.06 (10 analyses)	2.17	±0.14 (16 analyses)	1.77	±0.15

TABLE II. Sulfhydryl and Disulfide Contents of Two Strains of *T. hippicum* Expressed as the Cysteine Equivalents.

Normal strain				Arsenic-resistant strain			
Sulfhydryl mg/g	Combined mg/g	Disulfide mg/g		Sulfhydryl mg/g	Combined mg/g	Disulfide mg/g	
Mean	Standard error of mean	Difference	Standard error of diff.	Mean	Standard error of mean	Difference	Standard error of diff.
0.31	±0.01 (8 analyses)	1.49	±0.10 (22 analyses)	1.18	±0.10	0.27	±0.01 (8 analyses)	2.37	±0.04 (16 analyses)	2.10	±0.04

[4] Schmitt, F. O., and Skow, R. P., *Am. J. Physiol.*, 1935, **111**, 711.

[5] Barron, E. S. G., and Singer, T., *J. Biol. Chem.*, 1946, **157**, 221, 241.

[6] Reiner, L., Leonard, C. S., and Chao, S. S., *Arch. Intern. pharmacodynamie*, 1932, **43**, 199.

[7] Pedlow, J. T., and Reiner, L., *J. Pharm. and Exp. Therap.*, 1935, **55**, 179.

[8] Schueler, F. W., Chen, G., and Geiling, E. M. K., *J. Infect. Dis.*, 1947, **81**, 14.

from erythrocytes. The washed trypanosomes were next pipetted into 10 cc of 15% trichloroacetic acid, centrifuged, resuspended again in 10 cc of the trichloroacetic acid, and recentrifuged. To the protein residue were added 20 cc of anhydrous acetone, and the suspension was shaken for 5 minutes, whereupon 1 drop of concentrated hydrochloric acid was added with shaking and the mixture centrifuged. This was repeated, after which the acetone was drawn off under reduced pressure until the residue became pasty. It was then ground in a hot mortar until a fine powder was obtained. After drying 30 min. at 105°C. the sample was ready for analysis. These preparations were white or slightly buff colored. About 60 mg of dry protein were yielded by 10^{10} trypanosomes.

The analyses of the sulfhydryl and disulfide groups were carried out by the method of Mirsky and Anson,[9] with only such minor adaptations as are required for use with the spectrophotometer (Coleman Universal, Model 11).

Results. The values given in Tables I and II are expressed as the cysteine equivalents of sulfhydryl and disulfide in mg per g of dry protein. The values of the disulfide content reported are the differences between the two means of the original reduced sulfhydryl and the total (combined) sulfhydryl found after thioglycollate reduction of the protein.

Discussion. Although the mean of the sulfhydryl content of the arsenic-resistant strain of *T. equiperdum* is 25% greater than that of the normal strain, *the difference is not statistically significant.* On the other hand, the resistant strain of *T. hippicum* shows 13% less sulfhydryl than the normal strain, and this value has a moderate statistical significance (significant difference, $t=2.8$). More significant, however, are the differences in the disulfide contents of the normal and resistant strains. If it be assumed that the disulfide groups are sulfhydryl reserves which may be actively converted into sulfhydryl under the equilibrium displacements effected by the arsenical, the fact that the resistant strain contains only 31% as much disulfide as the normal strain, in the case of *T. equiperdum,* might seem to agree with reports that arsenic-fast strains of *T. equiperdum* bind less arsenic in the presence of glucose than normal strains.[7,10] Reiner *et al.*[6,7] warn that such differences in arsenic binding are probably the result of comparing two strains of trypanosomes not in the same degree of suppression. Furthermore, the differences shown in our results are too small to account for the high degree of resistance manifested toward certain arsenicals, the resistance factors of which may approach 200,[10] nor can any hypothesis based upon the sulfhydryl or disulfide content as yet explain the marked influence of the side chain upon the resistance factor. The fact that arsenic-resistant *T. hippicum* contains not less, but more, disulfide than the normal strain complicates even more the problem of establishing any direct relationship between the magnitude and state of the sulfhydryl system and arsenic-resistance. It can only be asserted that certain constitutional changes probably giving rise to arsenic-resistance also give rise to changes in the sulfhydryl system, but that the latter is not directly, at least, responsible for the drug fastness.

By calculation from the mean value of the sulfhydryl content of *T. equiperdum* there are 2.7×10^{-3} milliequivalents of sulfhydryl per gram of protein or approximately 1.6×10^{-4} milliequivalents per 10^{10} trypanosomes, capable of binding 59μg of arsenic or 78μg of arsenic as As_2O_3 (assuming only 2 binding valences per atom). This amounts to 4.8×10^{6} molecules of arsenical per trypanosome. These calculations are not inconsistent with the results of Reiner *et al.*[11] who report an average binding of 0.1 microequivalent of arsenic per 10^{10} trypanosomes or 4×10^{6} molecules per trypanosome.‡ The maximum arsenic uptake (phenylarsenoxide) reported by Eagle and Magnuson[10] in a series of experiments is calculated to be 83μg of arsenic

[9] Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1935, **18**, 307.

‡ Their figure of 6×10^6 is too high by a factor of 3/2, since one equivalent is ½ atom in most organic arsenicals.

[10] Eagle, H., and Magnuson, H. J., *J. Pharm. and Exp. Therap.*, 1944, **82**, 137.

per 10^{10} trypanosomes. Even higher degrees of binding have been reported[6,12] which are not commensurate with the quantity of sulfhydryl found by us. This might be construed as evidence for binding as a monothioarsenite or by some other means not involving the sulfhydryl group. However, it is more likely that the discrepancy may be attributed to conversion of disulfide to sulfhydryl during exposure or to a sulfhydryl level in the living trypanosome higher than that found in this investigation. The presence of substrate is important to the degree of binding,[7] and it is not unlikely that during centrifugation the closely packed trypanosomes suffer a relative glucose deficiency, which would yield low values of reduced sulfhydryl. That oxidation during the preparation of the sample was not grossly responsible for low values was tested by comparison with samples of wet protein whose dry weight equivalent was reasonably established by count.

Summary. Normal and arsenic-resistant strains of *T. equiperdum* do not differ significantly with respect to their sulfhydryl content, but the former shows a considerable surplus of disulfide groups. The sulfhydryl content of normal *T. hippicum* is not appreciably different from that of an arsenic-resistant strain, but the disulfide content of the arsenic-resistant strain is higher than that of the normal strain. It is concluded that these differences are not the cause of the resistance but may be rather the result of some cytological reconstitution which probably also gives rise to resistance. The sulfhydryl content is found to be of the same order as the degree of arsenic binding.

11 Reiner, L., Leonard, C. S., and Chao, S. S., *Arch. intern. pharmacodynamie,* 1932, **43**, 186.

12 Eagle, H., *J. Pharm. and Exp. Therap.,* 1945, **85**, 265.

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Agents Influencing Experimental Radiation Injury. Effects of Folic Acid and Pyridoxine.

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In a previous communication by one of us,[1] it was noted that a high mortality rate occurred among tumor bearing mice which were treated with X-rays for therapeutic purposes. The survival time even of successfully treated animals was short. It has been assumed that the animals might have succumbed due to either the absorption of the disintegrated tumor, or to same toxic substances produced by secondary, or scattered, radiation. The latter assumption was based on observations made from earlier experiments using the tissue culture technique.[2] Thus it was noticed that tissue fragments remaining in the medium in which they were irradiated needed less dosage for preventing their growth *in vitro,* than those which were washed in Tyrode solution and transferred to a fresh non-irradiated medium. It was concluded that some toxic substances might have been produced in the culture medium by radiation. Similar observations were made by other investigators.[3,4] The so-called "radiation sickness" occurring among human patients treated with X-rays may also be the result

1 Goldfeder, Anna, *Radiology,* 1945, **44**, 283.

2 Goldfeder, Anna, *Radiology,* 1938, **31**, 73; 1940, **35**, 210.

3 Luria, S. E., and Exner, F. M., *Proc. Nat. Acad. Sci.,* 1941, **27**, 370.

4 Evans, T. C., Slaughter, J. C., Little, E. P., and Failla, G., *Radiology,* 1942, **39**, 663.

of the action of similar toxic substances produced by ionizing radiation.

Attempts are being made to remove or counteract the undesirable effects produced by radiation. This report deals with such an attempt.

Materials and Methods. White mice, Swiss strain inbred, weighing between 20 and 22 g were used. These were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine. They were kept in metal cages and fed with Purina dog chow and water *ad libitum* for about 2 weeks prior to radiation, and their appearance, weight, and blood levels were noted.

It was necessary, first of all, to devise a procedure whereby the experimental object would receive a most uniform dose of radiation, and which would permit *reproducible* results within the least limits of error. It is known that biologic effects of radiation do not depend upon the physical calculated dose delivered, but upon the amount of radiation absorbed by the exposed object. Where the biologic object is very small, such as a drosophila egg or a tissue fragment, the amount of radiation delivered is equal to the amount absorbed. When, however, it concerns a larger object, the amount of radiation absorbed depends upon the size and shape of the object and upon the secondary radiation produced by back-scatter. To simplify matters a device was used in which the X-ray beam hits a single mouse placed in a separate compartment, thus permitting uniform radiation over the entire body surface. This procedure differs from those previously used by other investigators for similar experiments, in which batches of mice were exposed.[5,6,7] The procedure consists of the following:

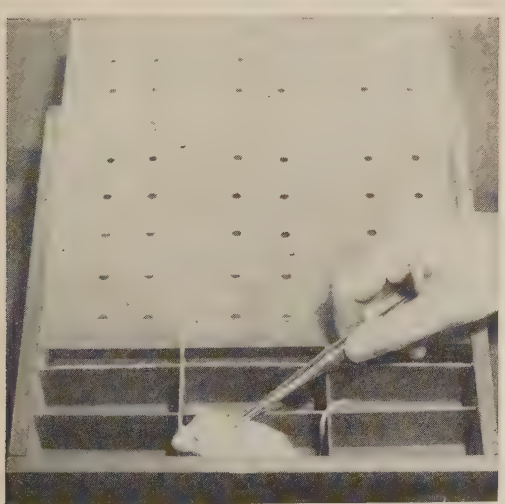

FIG. 1.
Wooden box with 24 compartments and perforated plastic cover in which mice were irradiated.

A square wooden box of white pine, 24x24 cm in size and 4 cm in depth, was divided by plastic partitions into 24 compartments of identical size and shape (7.5 cm in length, 2.7 cm in width, and 4 cm in depth). The box can be closed by a cellulose acetate cover in which small air holes of about 5 mm in diameter were made. One mouse was placed in each compartment and the cover was gradually slipped over the mice in the box (Fig. 1).

This box was placed directly in the useful beam of the X-ray tube at a distance of 80 cm. The distance from the target of the X-ray tube to the center of the body of the middle mouse was 82 cm, which was considered the target-tissue distance for the purpose of this experiment. A mouse at the corner of the box is necessarily at a slightly greater distance from the target, approximately 84 cm, but it receives only about 4% less radiation than the central animal. This degree of uniformity is satisfactory for the present purpose. The cellulose cover absorbs 2½% of the incident radiation, and this was taken into account in computing dosage.

The physical factors used were: 200 KV peak pulsating potential generator; 20 ma.; ½ mm Cu plus 1 mm Al filtration; half value layer 0.9 mm Cu; field 25x25 cm uniform at 82 cm target-skin distance. A dosage rate of 14 r/min. measured in air was obtained under the above conditions. The physical measurements were made by placing the ionization chamber through a hole in the center of one of the side walls of the box,

5 Henshaw, Paul S., *J. Nat. Cancer Inst.*, 1944, **4**, 477.

6 Ellinger, F., *Radiology*, 1945, **44**, 125.

7 Quastler, H., *The Am. J. Roent. and Radium Ther.*, 1945, **51**, 449.

Fig. 1. The ionization chamber was introduced parallel to the bottom of the box, perpendicular to the side, and was located in the center of the box. The measurements were taken with and without mice in the box. The difference between these measurements was approximately 2% more with the mice in the box. This figure is so small that it precludes any significant effect from scatter.

Doses were applied in single exposures, and based on measurements made in air.

Blood samples were obtained by the following technical procedure, which permitted the use of minimum amounts of blood without loss of accuracy. Short lengths of capillary tubing were calibrated with mercury to contain 5 cu mm. For taking blood samples the mouse was placed in a special device (Fig. 2). The tail of the mouse was first cleansed with alcohol and then wiped dry with cotton. The tip of the tail was snipped off with a pair of sharp scissors and the initial drop of blood wiped away. The tail was then gently massaged until a good sized drop accumulated and the capillary was placed in contact with the drop of blood. When full, the capillary tube was dropped into a small test tube containing 0.25 cc of a 1% acetic acid solution. This was gently shaken until a homogeneous suspension of the blood cells and the diluting fluid was obtained. A smear for a differential count was taken and the tail wiped dry. Lastly, a drop of collodion was applied to the tip of the tail to stop any further bleeding.

FIG. 2.
Mouse holder, and 5 cu mm capillary tube for blood samples.

The blood counts were done in the regular manner with the Bright-Line Improved Neubauer blood counting chamber, using the dilution factor of 500 to give the number of white blood cells in 1 cu mm of blood.

Pyridoxine and folic acid were chosen as the first test agents for the following reasons: The former has been widely used for some time clinically and shown to have a beneficial effect in preventing nausea of X-ray treated cases;[8,9,10] (the mechanism of its action is still obscure). The folic acid (*L. casei* factor) proved to have a beneficial effect in cases of sprue,[11,12] and in some degree on leucopenia. Since diarrhea and leucopenia are also symptoms of radiation sickness, it was of interest to test the efficacy of folic acid in these respects.

In evaluating the effects of irradiation produced by a given dosage, the following criteria have been used: (1) weight of the animals and their general appearance, (2) peripheral blood levels, (3) survival time in days following irradiation, and (4) morphological appearance of liver and spleen.

The observations made on W.B.C. counts and survival time of the irradiated, medicated, and non-medicated mice will be reported here.

Results and Discussion. The average white blood count of the control, non-ir-

[8] Scott, L. O., and Tarleton, G. J., *Radiology*, 1946, **47**, 386.

[9] Maxfield, J. R., McIlevain, A. J., and Robertson, J. W., *Radiology*, 1943, **41**, 383.

[10] Shervon, L. M., *Brit. J. Radiology*, 1946, **19**, 369.

[11] Watson, C. J., Serbell, W. H., Kelevey, J. L., and Daft, F. S., *Am. J. Med. Sci.*, 1945, **210**, 463.

[12] Hernadez, R. L., and Spies, T. D., *Am. J. Radiology and Radium Therapy*, 1946, **56**, 337.

TABLE I.
Effect of 200 r and 500 r on Survival Time and W.B.C. Count of White Mice.

	200 r	500 r
Lowest avg W.B.C. count	8000	1000
Duration of falling W.B.C.	2 days	10 days
Period of recovery	5 "	38 "
Deaths in leucopenic phase	0%	45%
Occurrence of first death	28 days	8 days
Period of 50% mortality	39 "	21 "

TABLE II.
Effect of Folic Acid on the Survival Time and W.B.C. Count of Mice Irradiated with 350 r.

	Medicated mice		
	Oral folic acid	Intramuscular folic acid	Control mice
Lowest avg W.B.C.	3,000	2,000	2,000
Duration of falling W.B.C. count	4 days	3 days	9 days
Period of recovery (W.B.C. normal avg 15,000)	22 "	25 "	23 "
Deaths in leucopenic phase	0%	2%	26%
Occurrence of first death	30 days	24 days	13 days
Period of 50% mortality	53 "	100 "	43 "

radiated mice used in these experiments was found to lie within 15,000-19,000. In order to ascertain the amount of radiation giving the most clear-cut results, preliminary experiments were carried out. With less than 200 r no consistent changes in blood count or any definite diminution in survival time within 4-6 weeks following radiation could be noted.

A group of 20 mice giving an average W.B.C. count of 17,000 was exposed to 200 r; the W.B.C. count fell to 8,000 on the second day following irradiation, and returned to normal levels (taking the average of 15,000 W.B.C. for the mouse) on the 5th day. None of the animals died in the leucopenic phase; the first deaths occurring on the 28th day after radiation; 50% had died on the 39th day. Table I summarizes the results.

A dose of 500 r applied to a group of 20 mice resulted in severe roentgen injury. The W.B.C. counts fell to very low levels. The leucopenia persisted longer than after a dose of 200 r, and resulted in a considerable proportion of deaths during this phase. The mice commenced to die at the end of the first week, and the 50% mortality fell on the 21st day. Table I summarizes the results.

It was considered that the acute trauma of this large dose resulted in dissolution of the experimental animals too soon for any possible benefit from medications to become apparent; for this reason an intermediate dose of 350 r was accepted as a standard for this investigation.

*Experiment with Folic Acid.** Four groups, 12 mice in each, were exposed to 350 r. As it was noted from preliminary experiments, 350 r is apparently sufficient to produce a sharp reaction with marked leucopenia during which a few animals die; most of the irradiated animals, however, survive long enough to show the more chronic radiation injury, and the effect of drugs on their survival.

One group was given 20 γ of folic acid orally daily following irradiation; another was given 15 γ of folic acid by intramuscular injection; and along with each of these treated groups a control group of animals was irradiated but no medication given.

In Table II are recorded the average results obtained from the experiments with folic acid.

The daily injections of 15 γ of folic acid for a period of 22 days (7 days prior to radiation and 15 days after radiation), each mouse

* Dr. Stanton M. Hardy, Medical Director of Lederle Laboratories, supplied the synthetic folic acid, under the commercial name, "Folvite." This is equivalent to 15 mg/cc of folic acid. This was diluted with saline solution to make 15 gammas in 0.1 cc for intramuscular injections.

TABLE III.
Effect of Pyridoxine on the Survival Time and W.B.C. Count of Mice Irradiated with 350 r.

	Medicated mice		
	Pyridoxine pre-radiation	Pyridoxine post-rad.	Control mice
Lowest avg. W.B.C. count	1800	2000	2500
Duration of falling W.B.C. count	6 days	4 days	10 days
Period of recovery	14 "	25 "	22 "
Deaths in leucopenic phase	17%	60%	85%
Occurrence of first death	9 days	2 days	3 days
Period of 50% mortality	100 "	16 "	14 "

thus receiving a total of 330 γ, greatly prolonged the average survival time of irradiated mice. This prolongation was evidenced by the fact that no animals died after the 25th post-radiation day. This drug had no obvious effect on the hematologic response, although it decreased the mortality rate in the leucopenic phase and afforded significant protection from delayed X-ray death after recovery of the leucopoietic system. This effect, however, was not manifest when folic acid was administered orally.

Experiment with Pyridoxine Hydrochloride. Pyridoxine hydrochloride (Squibb, for parenteral use), was used as a protective agent in 3 groups of 12 mice each, irradiated with 350 r. In one group, 50 γ of the drug were administered by intramuscular injection for 7 days prior to irradiation, and 13 days after irradiation, each mouse thus receiving a total of 1,000 γ; in a second group administration was begun after irradiation, and the third group was used as irradiated unmedicated control. The average results obtained from this experiment are summarized in Table III.

Analysis of the data recorded in Table III reveals that pyridoxine hydrochloride greatly prolongs the average survival time of irradiated mice. The protective effect is not produced unless the drug is administered before irradiation. This is evidenced in the 50% mortality rate occurring within 100 days, as compared to 16 days for the post-radiation treated mice, and that of 14 days for control irradiated, unmedicated mice. Pyridoxine does not appear to influence the onset, severity, or duration of the leucopenia, but gives a definite protection against the delayed roentgen injury, as no animals died after recovery of the leucopoietic system. In this respect, pyridoxine hydrochloride resembles folic acid.

An average loss of weight of 1 to 2 g per irradiated mouse, experimental and control, was noticed during the leucopenic phase. This loss of weight, however, was regained within 3-4 days by the surviving mice.

Both pyridoxine and folic acid seemed to have diminished and in some cases prevented diarrhea of the irradiated mice. Only about 10% showed this symptom within 3 days following radiation; while all of the irradiated unmedicated mice had diarrhea. The usual ragged appearance of the hair of irradiated animals was not noticed on those medicated either with pyridoxine or with folic acid. In particular, the hair of the mice treated with pyridoxine appeared more sleek and luxurious than those treated with folic acid.

Since the results obtained from the preliminary experiments recorded above seemed to indicate that folic acid and pyridoxine hydrochloride prolonged the survival time of irradiated mice, both these agents were tested on an additional 124 mice divided into three groups, using the same technique as previously described.

The results are summarized in the accompanying curves, Fig. 3 and Fig. 4, in which the average daily W.B.C. count and the proportion of animals surviving were plotted against time in days for a period of 100 days.

As can be noted in the graph, Fig. 3, of 32 mice treated with folic acid, 20 (62%) are still alive; and of 24 mice treated with pyridoxine hydrochloride 14 are living (58%), as compared to 68 controls, irradiated non-medicated mice, of which 13 (19%) are alive.

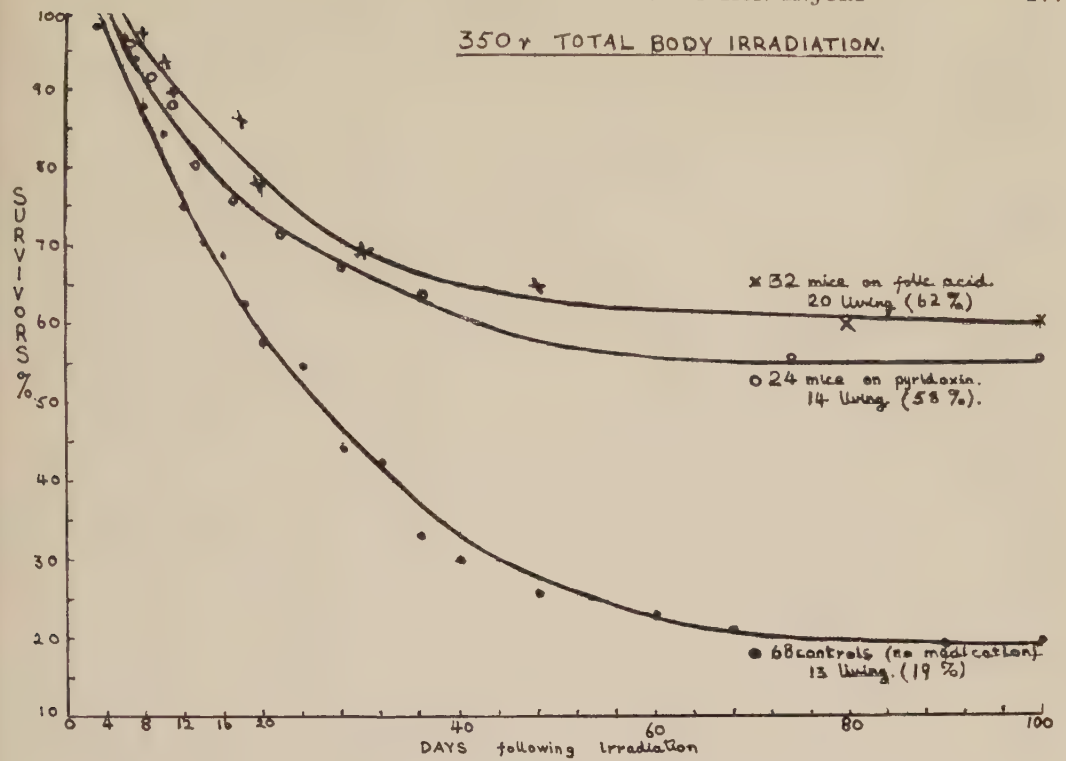

FIG. 3.

Curves showing % of survivors plotted against days following irradiation with 350 r of control mice, and mice medicated with folic acid, and pyridoxine.

From the experimental data reported here it might be concluded that folic acid and pyridoxine hydrochloride extend the survival time of mice exposed to 350 r.

Regarding the observations made in this study, reference is being made to 2 most recent publications along this line. In one, protective action of desoxycorticosterone acetate against X-ray-induced liver changes in mice was reported by Ellinger.[13] In the other, a reduction in the mortality rate of irradiated dogs treated with a flavonone, rutine, was reported by Rekers and Field.[14] To quote from these authors, "following irradiation of dogs with 350 r, 16 of 25 (64%) of the irradiated dogs succumbed within 13 to 30 days, whereas only 3 of 25 (10%) rutine-treated dogs died 16, 28, and 31 days post-radiation." These investigators attributed the beneficial effect in preventing capillary fragility, usually caused by radiation, to rutine. This effect was evidenced by the lack of pulmonary and intestinal hemorrhages in the medicated dogs, in contrast to the hemorrhagic condition noticed in irradiated, unmedicated dogs.

Possibly the beneficial effect of folic acid and pyridoxine hydrochloride reported in this paper, bears some relationship to the mechanisms of action of the preparations used by the other two investigators. This may be ascertained in our future histological studies of the internal organs of the animals used in our experiment, a great number of which are still alive.

Summary and Conclusion. White mice, exposed to X-radiation, given a medium dose of 350 r in a single exposure was found satisfactory under the experimental conditions described in the text. Folic acid and pyridoxine hydrochloride were used as possible protective agents. No appreciable effect of these drugs on the hemopoietic system was noted. On the other hand, it was found that

[13] Ellinger, F., *Science,* 1946, **104**, 502; PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 31.

[14] Rekers, P., and Field, J. P., *Science,* 1948, **107**, 16.

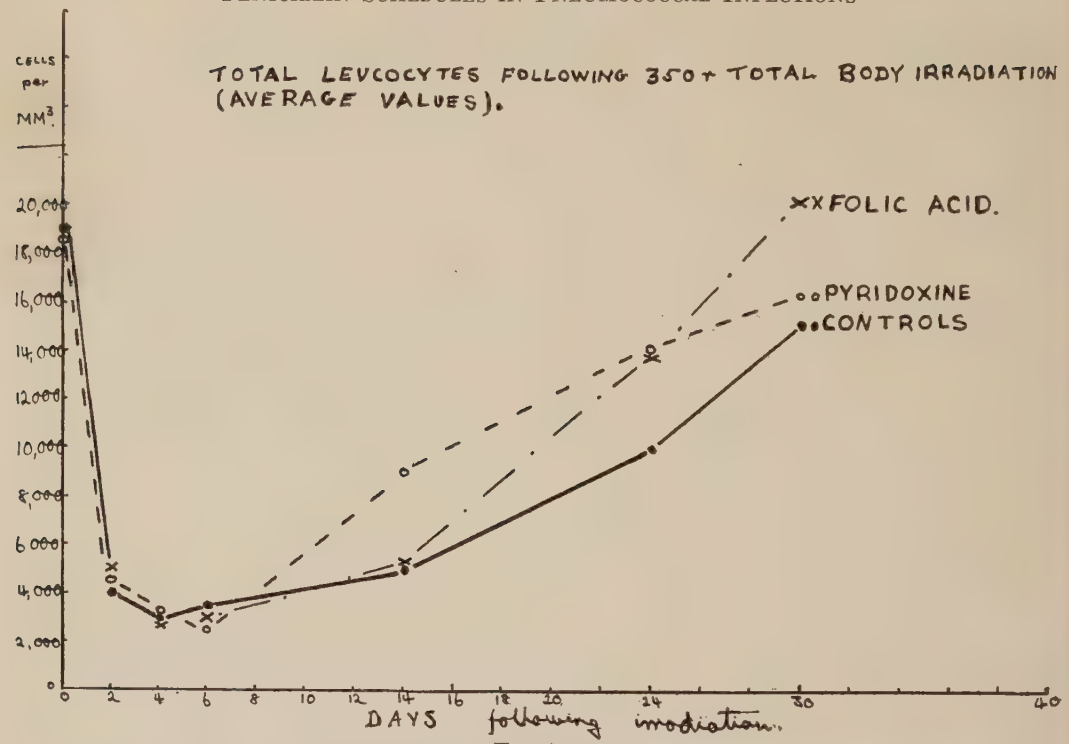

FIG. 4.

Curves showing average total W.B.C. counts plotted against days following irradiation with 350 r of control mice, and mice medicated with folic acid, and with pyridoxine.

15 γ of folic acid injected daily during a period of 7 days prior to radiation and 15 days after radiation prolonged significantly the survival time of the irradiated mice. Similar observations were made with pyridoxine hydrochloride. Thus, irradiated mice injected with 50 γ of pyridoxine hydrochloride intramuscularly daily 7 days prior to radiation and 13 days post-radiation, extended significantly the life span of the irradiated mice, as compared with the irradiated, unmedicated mice.

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Comparative Effectiveness of Two Penicillin Treatment Schedules in Pneumococcal Infections of Mice.

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The methods currently employed for penicillin therapy are based largely on the principle that adequate blood levels of the antibiotic must be constantly maintained throughout the period of treatment. Consequently, since penicillin is rapidly excreted after its injection in aqueous solution, repeated doses at 3-hourly intervals are usually given to re-

plenish the blood concentration. The demands of this therapeutic program impose a heavy burden on the nursing staffs of hospitals and create difficult problems for the practitioner who is treating patients at home, while the repeated injections are painful to the patient and disturb his rest. For these reasons attention lately has been turned to the question whether there is an essential need for constantly elevated blood levels of penicillin and whether injections at less frequent intervals may be successfully employed.

The observations of Tillett, Cambier and McCormack[1] were the first to suggest that the antibacterial effect of penicillin might persist for longer periods of time than was indicated by measurements of penicillin concentration in the blood, and that the wider spacing of injections might be safely attempted in the treatment of lobar pneumonia. These results were confirmed experimentally by Jawetz,[2] who treated hemolytic streptococcal infections in mice and demonstrated that the antibacterial effect of penicillin persisted for as long as 8 hours, while the blood concentration was measurable for only one hour. In addition, White, Lee, and Alverson[3] showed that mice infected with hemolytic streptococci could be cured with a single large dose of penicillin given orally. More recently Zubrod[4] determined the effect of different dosage schedules of penicillin upon hemolytic streptococcal infections in mice and showed that the controlling factor in survival rate was the total dosage of penicillin and not the interval at which it was administered.

The present report deals with the effect of penicillin on pneumococcal infections in mice, in which the same daily doses of the antibiotic were administered by 2 different treatment schedules. The work was carried out as a controlled laboratory experiment to complement clinical studies on the penicillin therapy of pneumococcal pneumonia in human subjects.

Experimental. Swiss mice of both sexes weighing from 15 to 18 g were inoculated intraperitoneally with 1,000 LD50 doses of a standard laboratory strain of Type I pneumococcus. Two hours after infection treatment was begun with crystalline sodium penicillin G* dissolved in a physiological solution of sodium chloride and injected subcutaneously in volumes of 0.2 to 0.5 cc, depending upon the dose employed. Groups of 20 mice were treated for 4 days with total daily doses of penicillin ranging from 20 to 140 units. Ten mice of each group received half of the daily dose at 12 hour intervals while the remaining 10 mice received one-eighth of the daily dose every 3 hours. One group of 10 mice was left untreated to serve as controls. As an additional control on the possible effect of the larger initial doses of penicillin in the animals treated twice daily, groups of 5 mice were given a single injection of penicillin ranging from 10 to 70 units. Finally, in order to ascertain whether a cure could be effected with a single massive dose of the drug, additional groups of 5 mice were given one injection of 100, 200 or 1,000 units of penicillin. The animals were observed for a period of 2 weeks following treatment at the end of which time the final mortality rates were recorded.

The results are shown in Table I and may be summarized as follows:

1. The minimal 100% curative dose of penicillin was 140 units daily regardless of whether this was administered in 2 equal parts at 12-hourly intervals or in 8 equal parts at 3-hourly intervals.

2. There were no apparently significant differences in the mortality rates among mice treated twice daily and 8 times daily with equivalent doses of penicillin less than 140 units.

3. It was possible to effect a 100% cure with a single injection of 1,000 units of penicillin.

The comparative PD50 doses of penicillin

[1] Tillett, W. S., Cambier, M. J., and McCormack, J. E., *Bull. N. Y. Acad. Med.*, 1944, **20**, 142.

[2] Jawetz, E., *Arch. Int. Med.*, 1946, **77**, 1.

[3] White, H. J., Lee, M. E., and Alverson, C., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 35.

[4] Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 400.

* Throughout the remainder of this paper the word "penicillin" refers only to crystalline penicillin G.

TABLE I.
Comparison of Penicillin Treatment Schedules in Pneumococcal Infection of Mice.

Penicillin units per day	Frequency of penicillin injections		
	Single inj.	Every 12 hr	Every 3 hr
1000	5/5*		
200	4/5		
140		9/9	10/10
120		7/10	9/10
100	2/5	9/10	6/9
80		9/10	8/10
70	3/5		
60	2/5	5/10	4/10
50	0/5		
40	0/5	2/10	1/10
30	0/5		
20	0/5	2/10	0/10
None		0/10	

* Numerator indicates number of mice surviving, denominator indicates total number of mice.

administered twice daily and every 8 hours were calculated according to the method of Reed and Muench.[5] The PD50 dose of penicillin was 60 units daily when given every 12 hours and 72 units daily when given every 3 hours. Although this would seem to indicate some advantage when the drug was administered twice a day in larger individual doses, the difference is not statistically significant.

Discussion. It must be pointed out that this experiment determined the minimal curative and PD50 doses of penicillin only for an arbitrarily selected treatment period of 4 days. What constitutes the absolute minimal effective dose of penicillin would appear, however, to depend mainly on 2 factors—the size of the daily dose and the duration of treatment—which bear some reciprocal relationship to one another. In other words, the larger the daily dose the shorter the time required for effective treatment. This is borne out by the fact that a single injection of penicillin may be curative, provided that the dose is large enough.

The outstanding feature of the results obtained under the conditions of this experiment is that the effectiveness of penicillin therapy appeared to depend more upon the total daily dose of the drug than upon the intervals at which it was administered. Our findings in the treatment of pneumococcal infections in mice are therefore entirely in accord with those of Jawetz[2] and Zubrod[4] for streptococcal infections in the same animal. It thus becomes evident, as suggested by Bigger,[6] that the mode of action of penicillin in controlling bacterial infection is a complex mechanism which cannot be explained simply on the basis of a constant effective concentration of the antibiotic. As one part of this mechanism the enhanced antibacterial effect of a sudden large increase in the level of penicillin may possibly counterbalance its virtual disappearance from the blood during prolonged intervals after administration, but how this is brought about and what other factors may be involved, especially with regard to the defensive reactions of the infected host, cannot be stated at present. Nevertheless it is clear that the antibacterial effect of penicillin persists long after measurable quantities are known to have disappeared from the blood[2,7] and that, under certain conditions, daily doses given in 2 injections have a therapeutic action equivalent to the same daily doses divided into 8 injections. These findings illustrate that within limits one may depart from the therapeutic principle of a constantly maintained level of penicillin and suggest a clinical application in the treatment of human infections.

Summary. The therapeutic effects of penicillin injected every 12 hours into mice infected with pneumococci were compared with the effects of similar daily doses divided into 8 injections at intervals of 3 hours.

With both treatment schedules the 100% curative daily dose and the PD50 daily dose were found to be similar.

The significance of these findings is discussed.

[5] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

[6] Bigger, J. W., *Lancet*, 1944, **2**, 497.

[7] Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

16276

Effect of Glucose, Peptone, and Salts on Streptomycin Activity.*†

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Among the factors which influence the antibacterial activity of streptomycin, the composition of the medium occupies a prominent place.[1,2] The influence of reaction and the effect of glucose, peptone, and of various salts have received special consideration.

Several explanations have been or can be suggested to account for the repressive action upon streptomycin potency by the lowering of the pH of the medium: 1. streptomycin is largely active in a free basic or dissociated state and to a lesser extent in the form of a salt; 2. the active part of the streptomycin molecule can largely be neutralized by an acid; 3. the streptomycin base and the hydrogen ions compete on the surface of the bacterial cell for the active centers; 4. dissociation of the streptomycin salt is repressed by a readily dissociated mineral salt.[3-8]

The effect of glucose in lowering the potency of streptomycin has been explained as due to the reducing properties of the sugar, to the production of an acid by the fermentation of the carbohydrates by the test bacteria, or to certain other factors such as the effect on the growth of the organism which would make it more resistant to the action of streptomycin.[4,6,7,9] The effect of certain peptones[2] and other organic compounds, such as sodium glycolate,[1] cysteine, and methionine, in lowering the activity of streptomycin has been explained by a reduction of the oxidation-reduction of the medium, by interaction with the active grouping of the antibiotic, or by some other mechanism.

Various theories have also been postulated to explain the effect of salts in reducing the potency of streptomycin. Loo *et al.*[5] noted that the addition of certain inorganic salts to streptomycin solutions caused a marked increase in activity of the antibiotic; in the presence of 0.1 *M* phosphate buffer, however, the effect of the salts was minimized, except at high concentrations. On the other hand, Klein and Kimmelman[10] reported that sodium chloride produced an antagonistic effect upon streptomycin activity. Berkman *et al.*[11] also observed a decrease in activity of streptomycin upon the addition of salts to nutrient broth.

In view of the importance of these constituents of the medium in determining the activity of streptomycin, a study was undertaken of the "pH effect," the "glucose effect," the "peptone effect," and the "salt effect."

Methods. Unless otherwise stated, *Escherichia coli* 9637 was used as a test organism.

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

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1 Donovick, R., and Rake, G., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 224.

2 Lenert, T. K., and Hobby, G. L., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 235, 242.

3 Waksman, S. A., Bugie, E., and Schatz, A., *Proc. Staff Meet. Mayo Clinic*, 1944, **19**, 537.

4 Waksman, S. A., and Schatz, A., *J. Am. Pharm. Assn., Sci. Ed.*, 1945, **34**, 273.

5 Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., Mcquire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, 1945, **50**, 701.

6 Geiger, W. B., Green, S. R., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 187.

7 Abraham, E. P., and Duthie, E. S., *Lancet*, 1946, **140**, 455.

8 Wolinsky, E., and Steenken, W., Jr., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 162.

9 Sykes, G., and Lumb, M., *Nature*, 1946, **168**, 271.

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The inoculum was prepared by growing the organism on nutrient agar for 24 hours, removing with sterile tap water, centrifuging, and suspending in water. The turbidity of the suspension was standardized by use of a Cenco-Sheard-Sanford Photelometer; when 0.1 ml of the suspension was inoculated into 4.9-ml portions of medium, an approximate count of 40 million bacterial cells per milliliter was obtained.

The culture tubes immediately after inoculation showed no absorption of light, since the instrument is not sensitive to suspensions containing fewer than 100 million organisms per ml. As growth proceeded, the medium became turbid. The relationship between turbidity and numbers of cells proved to be a straight line function; suspensions having a turbidity of 13, 22, 38, 59 and 65 contained 200, 410, 1,220, 2,520, and 2,900 million cells per ml, respectively.

The glucose and nitrogen sources were sterilized separately. The initial pH of medium was 6.8. The nutrient broth used as the basic medium contained 0.3% meat extract and 0.5% peptone.

Effect of glucose and peptone. In preliminary experiments, the antibacterial potency of streptomycin was found to decrease in the presence of glucose in the medium. The presence of glucose (Table I) caused a decrease in the pH value of the culture after 24 hours of incubation, giving a final pH of 4.7, 5.6, and 6.5 with 0.5, 0.1, and 0.025% respectively. These sugar concentrations allowed a normal growth of *E. coli* even in the presence of 10 μg of streptomycin per ml. When concentrations of only 0.01 and 0.005% glucose were present, however, only a slight change in reaction occurred; these concentrations exerted no effect upon growth inhibition of *E. coli* by streptomycin.

TABLE I.
Effect of Glucose upon Antibacterial Activity of Streptomycin.
E. coli used as test organism; cultures incubated 24 hours at 28°C.

Glucose, %	Streptomycin, μg/ml	Turbidity	Final pH of culture
0	0	+++	6.9
0	10	0	6.8
.50	0	+++	4.7
.50	10	+++	4.8
.10	0	+++	5.6
.10	10	+++	5.5
.025	0	+++	6.5
.025	10	++	6.3
.010	0	+++	6.7
.010	10	0	6.4
.005	0	+++	6.7
.005	10	0	6.6

To determine whether the nitrogen source in the medium influenced the effect of glucose on streptomycin activity, the peptone in the medium was replaced by tryptone, and varying concentrations of glucose were added. The acidity produced in the presence of tryptone was comparable to that obtained in the peptone broth, 0.5% glucose giving a pH of 4.9 after 24 hours of incubation (Table II). Tryptone did not, however, exert any repressive effect upon growth inhibition of *E. coli* by streptomycin even in the presence of 2% glucose. These results point definitely to the fact that it was not the glucose as such nor the acidity produced from bacterial metabolism that was responsible for the reduction in effectiveness of streptomycin upon *E. coli* in the peptone broth.

A comparison was, therefore, made of a number of commercial preparations of peptones and other hydrolyzed proteins (Table III). Comparable acidities were obtained after 21 hours of incubation in all the tubes containing the various nitrogen sources, the pH values ranging from 4.4 to 4.9. Only peptone 383619, peptonized milk, and neopeptone favored the growth of *E. coli* in the glucose broth containing 10 μg of streptomycin per ml; normally, this organism is sensitive to 1 μg per ml of streptomycin. Regular inhibition of *E. coli* by streptomycin was obtained in the media containing tryptone, tryptose, proteose peptone, peptone 23368, and protone as sources of nitrogen. These results tend to indicate that neither the glucose nor the acidity produced in the metabolism of the sugar by the test bacterium was responsible for the reduction in streptomycin activity in the peptone medium.

Effect of salt. The above experiments pointed to the presence in some of the protein hydrolysates of another substance that interfered with the antibacterial action of

TABLE II.
Influence of Different Peptones upon Glucose Effect on Streptomycin.

Nitrogen source	Glucose, %	Streptomycin, μg/ml	Turbidity* 5 hr	Turbidity* 21 hr	Final pH
Tryptone	2.0	0	23	39	4.8
"	2.0	10	3	3	6.6
"	1.0	0	22	37	4.9
"	1.0	10	0	0	6.7
"	0.5	0	21	37	4.9
"	0.5	10	0	0	6.7
"	0.25	0	22	35	4.9
"	0.25	10	0	0	6.7
"	0.10	0	27	36	5.0
"	0.10	10	0	0	6.6
Peptone	1.0	0	18	34	4.6
"	1.0	10	15	35	4.7

* Absorption of light in per cent.

TABLE III.
Influence of Peptones and Protein Hydrolysates upon Glucose Effect on Streptomycin.
All media contained 1% glucose and 0.3% meat extract; cultures were incubated for 21 hours at 28°C.

Protein hydrolysate*	Streptomycin, μg/ml	Turbidity	Final pH
Peptone 383619	0	41	4.4
" 383619	10	26	4.7
" 23368	0	43	4.4
" 23368	10	0	6.7
Tryptone	0	44	4.8
"	10	0	6.7
Tryptose	0	48	4.9
"	10	0	7.1
Peptonized milk	0	52	4.9
" "	10	40	5.0
Peptinum siccum	0	30	4.5
" "	10	0	6.8
Proteose peptone	0	38	4.5
" "	10	0	6.7
Neopeptone	0	40	4.6
"	10	11	4.9
Protone	0	45	4.9
"	10	0	—

* All hydrolysates were used in 0.5% concentration, except peptonized milk of which 1.5% was added.

streptomycin. The salt concentration appeared to be a possible factor, since various salts were found to exert a depressive effect upon the potency of streptomycin.

A chloride analysis of the various peptone preparations revealed significant differences in salt content. Peptone 23368, which did not prove favorable to the glucose effect upon streptomycin activity, contained the least amount of chloride, namely 1.52%, and tryptone contained 1.58% chloride. Neopeptone, however, contained 8.12% chloride; the peptonized milk contained 1.65% chloride, but since 1.5% of it was used as compared to 0.5% for the other peptones, there was three times this salt concentration in the medium.

The influence of salt on the activity of streptomycin in media containing peptone and tryptone was, therefore, investigated. The addition of sodium chloride to these media, with and without glucose, produced good growth of *E. coli* even in the presence of 10 μg of streptomycin per ml (Table IV). In the absence of sodium chloride, the various peptones, with the exception of 383611, and tryptone did not inhibit the antibacterial action of streptomycin. The addition of 0.5% sodium chloride resulted in a neutralizing effect of the streptomycin. These results tend to substantiate the above conclusion that glucose is not the agent responsible for the reduction of streptomycin activity but that the effect is due to the salt content of the medium.

The cultures of *E. coli* in which growth took place in the presence of 10 μg/ml of streptomycin were analyzed for the possible development of resistant strains or for the possible destruction of some of the streptomycin. When these cultures were inoculated with nutrient broth containing streptomycin no growth occurred, indicating that the organism did not become resistant to this antibiotic. An analysis of the antibiotic potency of the tubes in which growth occurred in the presence of streptomycin (10 μg/ml), due to the glucose-salt effect, gave the theoretical concentration of the

TABLE IV.
Combined Effect of NaCl and Peptones on Streptomycin Activity in Glucose Media.
Cultures incubated for 22 hours at 28°C.

Peptone source	NaCl, 0.5%	Meat extract, 0.3%	Turbidity: Streptomycin, 0	Turbidity: 10 μg/ml, +
Peptone 327393	0	0	27	0
" 327393	+	0	19	19
" 327393	0	+	45	30
" 327393	+	+	47	38
" 23368	0	0	22	0
" 23368	+	0	23	20
" 23368	0	+	43	0
" 23368	+	+	41	37
" 383611	0	0	25	0
" 383611	+	0	22	21
" 383611	0	+	43	27
" 383611	+	+	44	35
Tryptone	0	0	30	0
"	+	+	37	29
"	0	+	45	0
"	+	+	48	38

TABLE V.
Effect of Salt upon Streptomycin Activity in Agar Media.
Cup method used; zone of inhibition measured in mm.*

Concentration of NaCl, %	Salt added to agar. Streptomycin, μg/ml: 5	10	50	Salt added to streptomycin solution. Streptomycin, 10 μg/ml
0	13.0	14.0	18.5	17.0
0.5	14.0	16.0	20.0	18.0
1.0	12.0	14.0	19.0	18.1
3.0	9.0	9.0	14.0	19.0

* Diameter of cup 9 mm.

antibiotic, thus proving that the streptomycin was not destroyed.

The salt phenomenon was shown to be effective not only for *E. coli* but also for *Bacillus subtilis* and *Proteus vulgaris*. Furthermore, other salts, such as magnesium chloride, sodium nitrate, and sodium sulfate, in *M/3* concentrations, also inhibited the antibacterial activity of streptomycin. These results are in accord with those obtained by other investigators. The salt effect was not neutralized by the addition of phosphate buffer or of serum to the medium.

When sodium chloride was added to nutrient agar, and the activity of streptomycin tested by the agar diffusion or cup method, it was found (Table V) that 0.5% salt actually caused an increase in the width of the zone of inhibition due to streptomycin; the addition of 3% salt to the agar resulted, however, in a marked reduction in the size of the zone. When salt was added to the streptomycin solution before it was placed in the cup, there was no neutralizing action, but an actual stimulating effect upon the potency of streptomycin.

These results indicate that the effect of salt is due not to a modification of the streptomycin but to a change in the conditions in the medium which influence the growth of the test organisms.

Summary. 1. The potency of streptomycin is greatly influenced by the composition of the medium, the salt concentration being most significant.

2. The inhibiting effect of glucose upon streptomycin activity is due to the specific nature of the organic nitrogenous compounds

in the medium and to the salt concentration.

3. The effect of salt is exerted not upon the streptomycin itself but upon the medium, which in turn influences the growth of the organism.

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Effect of Organic Acids on Streptomycin Activity.*†

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The antibacterial potency of streptomycin is still largely measured by its inhibiting effect upon the growth of certain bacteria in artificial culture media, under given conditions of cultivation. The activity of the antibiotic thus measured is influenced by a great many factors. Chief among these is the presence of certain organic and inorganic constituents in the medium,[1] the presence of certain reducing substances,[2,3] the nature of the test organism, its age and number of viable cells used.[4] In addition to these, the presence of certain organic acids that may be formed as intermediary metabolic products in the nutrition of various bacteria may also affect the activity of streptomycin. A study of the last phenomenon forms the subject of this report.

Escherichia coli ATTC 9637 was used as the test organism. Its growth in various media was determined by means of a Cenco-Sheard-Sanford Photelometer. Immediately after inoculation, the culture tubes gave a reading of zero turbidity. The final turbidity was expressed in terms of per cent of light absorption.

The organic acids used in these studies were neutralized to pH 7.0, sterilized by filtration through Mandler candles, and added to the medium to give a final concentration of 1%. The streptomycin solution was sterilized by heating at 60°C for 30 minutes. The basic nutrient broth used in these experiments contained 0.5% peptone and 0.3% meat extract.

The influence of different organic acids as compared to that of sugars and glycerol upon streptomycin activity was at first determined (Table I). The growth of *E. coli* in nutrient broth is usually inhibited by 1 μg of streptomycin per ml. When pyruvic or fumaric

TABLE I.
Effect of Various Carbon Sources in Medium upon Streptomycin Activity on *E. coli*.

Carbon source 1%	Turbidity* 4 hr Streptomycin, 0	4 hr Streptomycin, +	21 hr Streptomycin, 10 μg/ml 0	21 hr Streptomycin, 10 μg/ml +
Glucose	—	—	41	0
Lactose	14	0	36	0
Pyruvic acid	23	13	67	31
Fumaric acid	14	12	53	30
Formic acid	0	0	15	12
Succinic acid	8	6	30	7
Glycerol	8	0	42	0
Maleic acid	10	6	40	12
Malonic acid	8	6	31	6
Glycerophosphoric acid	7	0	33	0
Acetic acid	6	0	34	0
Propionic acid	5	0	30	0
Lactic acid	9	0	52	0

* Absorption of light in per cent.

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

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TABLE II.
Effect of Different Concentrations of Streptomycin upon Growth of *E. coli* in Presence of Pyruvic or Fumaric Acids.

Carbon source 1%	Streptomycin, μg/ml	Turbidity 5 hr	8 hr	17 hr	22 hr
Pyruvic acid	0	14	19	50	48
	10	7	14	33	34
	25	2	5	23	27
	50	0	0	0	2
	100	0	0	0	0
Fumaric acid	0	10	25	49	49
	10	9	20	40	41
	25	6	16	29	32
	50	1	3	15	16
	100	0	0	0	0

TABLE III.
Influence of Nitrogen Sources upon Effect of Organic Acids and Other Carbon Compounds upon Streptomycin Activity.*

Additional material	Casein hydrolysate		Asparagine Streptomycin, 10 μg/ml		Tryptone	
	0	+	0	+	0	+
None	20	0	2	0	22	0
Glucose	38	0	2	0	39	0
Lactose	33	0	0	0	30	0
Formic acid	4	3	4	0	13	6
Maleic acid	29	22	5	5	21	10
Malonic acid	44	10	11	0	21	4
Malic acid	51	15	16	0	40	18
Succinic acid	38	10	12	0	30	10
Pyruvic acid	48	37	50	2	42	40
Glycerol	37	0	2	0	29	0
Fumaric acid	35	22	17	2	36	19
Lactic acid	57	0	9	0	36	0

* *E. coli* used as test organism; incubation 20 hours at 28°C.

acid was added to the medium good growth was obtained even in the presence of 10 μg/ml of the antibiotic. Succinic, formic, maleic, and malonic acids also exerted a certain degree of protection upon the bacteria against the antibacterial action of streptomycin. Lactose, glycerol, sodium glycerophosphate, lactate, and glucose had, however, no effect upon streptomycin activity; the same was true of propionic and acetic acids.

The pyruvate and fumarate media in which *E. coli* grew in the presence of streptomycin were tested, by the agar cup method, for streptomycin concentration. The antibiotic was not reduced in potency. The possible development of bacterial strains resistant to streptomycin was also determined; the culture was found to remain sensitive to 2 μg/ml of streptomycin.

A study was made next of the minimum concentration of streptomycin necessary to inhibit the growth of *E. coli* in the presence of pyruvate and fumarate. Even in the presence of 50 μg/ml of streptomycin good growth was obtained in the peptone broth to which fumarate was added; there was no growth, however, with 100 μg/ml streptomycin (Table II). The effect of pyruvate was similar to that of fumarate, although quantitatively somewhat different. When the concentration of the fumarate was increased to 3%, growth occurred even in the presence of 150 μg/ml of streptomycin but none with 200 μg/ml.

The addition of organic acids and sugars to tryptone or casein hydrolysate broth gave results similar to those obtained with the peptone broth (Table III). When asparagine

was used as a source of nitrogen, slight growth of *E. coli* occurred in the presence of streptomycin with maleic acid, and a trace of growth with pyruvic and fumaric acids; the other carbon sources did not antagonize the action of streptomycin upon the growth of *E. coli*. This indicated the possibility that the complex nitrogenous materials contained some substance which was necessary for the utilization of the pyruvate and fumarate in the presence of streptomycin; this substance could not be derived from asparagine.

In an effort to elucidate the possible nature of the substance which favored the neutralizing effect of pyruvate and which was absent in asparagine, yeast extract and a vitamin mixture containing B_1, B_2, B_6, calcium pantothenate, folic acid, nicotinamide, para-aminobenzoic acid, and cocarboxylase were added to asparagine-pyruvate broth. Only the yeast extract proved effective in allowing the growth of *E. coli* in the presence of streptomycin (Table IV).

TABLE IV.
Effect of B Vitamins and Yeast Extract upon Antibacterial Activity of Streptomycin in Asparagine-Pyruvate Broth.
Asparagine 2.5 mg/ml, pyruvate 10 mg/ml.

Supplementary additions to broth	Mg/ml	Turbidity after 25 hrs Streptomycin, 10 μg/ml 0	+
Broth alone*		13	0
Pyruvate control		28	28
Yeast extract	2.5	51	17
" "	1.25	50	6
" "	0.63	52	8
Vitamin mixture		8	0

* No pyruvate.

The neutralizing effect of pyruvate and fumarate upon streptomycin was tested with other bacteria, including another strain of *E. coli* (No. 2), *Aerobacter aerogenes*, *Proteus vulgaris*, and *Staphylococcus aureus*. The action of pyruvate and fumarate upon streptomycin activity was found to vary with the organism (Table V). It was most marked with *P. vulgaris* and *E. coli*, and least with *A. aerogenes* and *S. aureus*. Similar variations were obtained with other organisms. *Mycobacterium tuberculosis* No. 607 showed some inhibition by fumarate with low concentrations of streptomycin and none with higher concentrations; the pyruvate showed no effect at all for this organism.

The results tend to show that certain organic acids have a definite neutralizing or antagonistic effect upon the antibacterial properties of streptomycin only as regards certain organisms. The salts of the dicarboxylic acids (fumaric, succinic, malic, and maleic), as well as pyruvic and formic acids, were most effective; they supported the growth of *E. coli* in the presence of bactericidal concentrations of streptomycin. The salts of acetic, lactic, and propionic acids, as well as sugars, such as glucose and lactose, did not influence the inhibiting action of streptomycin upon *E. coli*.

The presence of pyruvic and dicarboxylic acids does not result in the destruction of the antibiotic, since the concentration of streptomycin in the medium remained close to the theoretical. These organic acids do not favor the development of strains of bacteria resistant to the action of streptomycin. No irreversible combination is produced between the antibiotic and the organic acid. The only explanation for this effect may be looked for in the susceptibility of a specific enzyme system to the action of streptomycin, whereby it may be replaced, at least in the metabolism of certain bacteria, by another system in the presence of pyruvic or fumaric acid. That the effect of streptomycin upon susceptible bacteria may be due to interference with intermediary metabolism has been suggested.[6]

It may also be of interest to note here that the oxidation of benzoic acid by *Mycobacterium tuberculosis* No. 607 was inhibited by 10 μg/ml streptomycin, whereas even 100 μg had no effect upon this oxidation process by a resistant strain of this organism. On the other hand, 100 μg streptomycin had no effect upon the oxidation of pyruvic acid by the normal strain.[7]

The possibility that streptomycin interferes with the synthesis of amino acids, proteins, or carbohydrates and that the organic

5 Geiger, W. B., *Arch. Biochem.*, 1947,

6 Strauss, E., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 97.

7 Bernheim, F., and Fitzgerald, R. J., *Science*, 1947, **105**, 435.

TABLE V.
Effect of Pyruvate and Fumarate upon Antibacterial Activity of Streptomycin.
Incubation 17 hours at 28°C.

Carbon source	*E. coli*		*A. aerogenes*		*P. vulgaris*		*S. aureus*	
	0*	+	0	+	0	+	0	+
None	15	0	18	0	4	0	24	0
Pyruvic acid	36	29	40	4	36	26	44	13
Fumaric acid	40	23	46	6	47	42	22	6
Glucose	37	0	56	0	14	0	44	0

* 0 = no streptomycin; + = 4 μg/ml streptomycin.

acids may counteract the antibiotic effect by acting as essential metabolites in the synthesis of these compounds has been suggested.[5] Certain preliminary results obtained in oxidation experiments, by the use of the Thunberg and Warburg techniques, indicated that streptomycin does not affect metabolic reactions measured in terms of oxygen uptake or dehydrogenase activity; rather it appeared that streptomycin affects a synthetic process, and the effect on respiration is only a secondary phenomenon.

Summary. The addition of 1% pyruvate and fumarate to nutrient broth supported the growth of *E. coli* in the presence of 10 μg/ml of streptomycin. When the concentration of the acids was increased to 3%, growth took place even in the presence of 150 μg/ml of streptomycin. The streptomycin was not destroyed. The test bacteria growing in the presence of streptomycin and of the organic acids did not become resistant to streptomycin.

Salts of succinic, formic, malic, and maleic acids also exerted some antagonistic effect upon streptomycin. Lactose, as well as lactic, acetic, and propionic acids, glycerol, glycerophosphate, and glucose had no effect upon the growth-inhibiting action of streptomycin on *E. coli.*

Comparison of the antagonistic effect of pyruvic and fumaric acids against the streptomycin action upon various bacteria, showed considerable variation. *E. coli* and *P. vulgaris* were largely protected by the organic acids against the action of streptomycin, whereas *A. aerogenes* and *S. aureus* were only slightly affected.

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Urinary Excretion of 17-Ketosteroids by Normal Young Men During Starvation.*

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There are only a few reports indicating the effect of starvation on the urinary excretion of 17-ketosteroids. In most of these, owing

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to a number of complicating factors, it is impossible to assess the influence of the restricted food intake on the urinary excretion.

Dingemanse and her associates[1] found a marked decrease in the concentration of 17-ketosteroids in the urine of the inhabitants of the Netherlands during the German occupation. As they point out, although the content per unit volume was lower during the war than afterwards, the absolute amounts excreted in the two periods may not have been very different since the urine volume during the occupation was 1.5 to 2 times normal.

Salter *et al.*[2] noted a slight decrease in the mean urinary excretion of 17-ketosteroids among 48 repatriated American prisoners-of-war from the Pacific theater. All of these men had gynecomastia at one time or another. The biochemical manifestations of gynecomastia in these ex-P.O.W.'s were said to have been very similar to those observed in similar cases among civilians where the breast hypertrophy and tumor was presumably due to causes other than malnutrition. The papers by the Yale workers give the impression that the decreased 17-ketosteroid excretion is not a manifestation of starvation *per se*.

Chou and Wang[3] reported that the excretion of male sex hormones (as determined by the same general technique used in the 17-ketosteroid analysis) was markedly reduced in malnourished patients in proportion to the degree of undernutrition. However, factors other than starvation must have been operative in these cases since their well-nourished patients showed a rate of excretion of male sex hormones only 42% of the mean for their normal subjects.

Methods. The amount of total neutral 17-ketosteroids in the urine was determined by the micro-method of Miller and Mickelsen.[4] This involved the simultaneous hydrolysis of the urine with hydrochloric acid and extraction with carbon tetrachloride. After a series of washings with potassium hydroxide, hydrochloric acid and water, the carbon tetrachloride extract was evaporated to dryness *in vacuo*. The residue was dissolved in ethyl alcohol and then subjected to the usual Zimmermann reaction. In all cases duplicates were carried through from the beginning of the procedure and repeated when they failed to agree within 4%. All urine specimens were collected in bottles containing glacial acetic acid as a preservative. The analyses were started within 24 hours of the time of collection, or the sample was stored at −20°C until the analysis could be made.

Semi-starvation experiment. Thirty-four normal young men between 19 and 32 years of age voluntarily submitted to a semi-starvation experiment. During the 6-month period when they were maintained on an average daily intake of 1600 calories, they lost 24% of their original body weight. The diet simulated that available to the inhabitants of north-central Europe and was relatively adequate in all determinable factors other than calories and protein. Before the experiment started, the subjects were put through various physical and psychological tests in order to exclude those who deviated from normal. Other details of this experiment have been described elsewhere.[5]

A random group of 10 of these men was chosen for the study of the 17-ketosteroid excretion. The semi-starvation phase of the experiment was preceded by a 3-months control period. No urine samples were collected for these special analyses at that time. However, control samples were collected from some of the men on the 57th week of rehabilitation when the subjects were back to normal in all respects. Additional control urinary excretion values from a large group of similar young men were available for comparative purposes.

Urine samples were collected for the deter-

[1] Dingemanse, E., Huis in't Veld, L. G., and de Laat, B. M., *J. Clin. Endocrinol.*, 1946, **6**, 535.

[2] Salter, W. T., Klatskin, G., and Humm, F. D., *Am. J. Med. Sci.*, 1947, **213**, 31.

[3] Chou, C. Y., and Wang, C. W., *Chinese J. Physiol.*, 1939, **14**, 151.

[4] Miller, E. v. O., and Mickelsen, O., to be published.

[5] Henschel, A., Mickelsen, O., Taylor, H. L., and Keys, A., *Am. J. Physiol.*, 1947, **150**, 170; Keys, A., *J. Am. Diet. Assn.*, 1946, **22**, 582.

TABLE I.

Urinary Excretion of 17-Ketosteroids During Semi-starvation and Subsequent Rehabilitation. All values are expressed as mg of androsterone per 24 hours. The mean urine volumes as cc per 24 hrs are given. The standard deviations are given with the means.

Subject	S8	S24	R6	R57
1	10.8	10.2	17.6	12.6
9	10.8	9.8	12.8	15.0
12	3.3	8.7	10.9	11.7
104	7.7	6.9	9.8	11.2
122	7.0	6.4	9.6	10.3
Mean	7.92 ± 3.12	8.40 ± 1.70	12.14 ± 3.31	12.16 ± 1.79
23		4.7	10.7	13.0
26		7.7	18.2	10.1
109		8.0		11.0
130		11.5	5.8	17.5
232	8.6	8.8		
Mean (all subjects)	8.03 ± 2.80	8.27 ± 1.99	11.92 ± 4.18	12.49 ± 2.42
Urine vol. (all subjects)	1848 ± 759	3009 ± 1794	3838 ± 925	1380 ± 383

mination of the ketosteroid excretion at the following times: (1) the eighth week of starvation (S8) when the men had lost an average of 13% of their original body weight, (2) at the twenty-fourth week of semi-starvation (S24) when they had lost 24% of their original body weight, (3) at the sixth week of rehabilitation (R6) when the men had regained some of their lost weight and (4) at the 57th week of rehabilitation (R57) when they were back to normal in all respects.

Acute starvation experiment. Twelve normal young men were subjected to a 4½-day period when they received no food. Water was the only substance permitted by mouth during this period and it was unrestricted. Before and after this, these men were the subjects of a long-time experiment during which they received diets controlled as far as calories and thiamine were concerned. Throughout the starvation period, the physical activity of the subjects was maintained by standardized walking on a motor-driven treadmill. The work was of such intensity that a calculated caloric deficit of approximately 5000 calories per day was produced. The training factor involved in this high plane of activity was cancelled out since these men had done the same amount of work for a week or more preceding the fast.

Urine samples for the estimation of the 17-ketosteroid excretion were collected on the third and fourth days of the fast. The control samples were secured some months later. These values agreed with those secured a number of months preceding the experiment. The validity of these control samples has been independently proven by the constancy of the 24-hour urinary excretion of total, neutral 17-ketosteroids over periods as long as a year or more.[6,7]

Results—semi-starvation experiment. The excretion of total, neutral 17-ketosteroids for the 5 men studied at S8 averaged 7.9 mg per 24 hours (Table I). This is considerably below the mean excretion level of 11.3 mg observed for normal young men in this laboratory[7] as well as the 12.2 mg for these men at the 57th week of rehabilitation. Each subject at S8 showed a lower excretion than that during the recovery period. In fact subject 12 showed an excretion level considerably below any observed by us in normal young men, while the values for the rest of the subjects were in the lower normal range.

At S24, the 17-ketosteroid excretion of the above group showed no essential change except for subject 12 whose value increased considerably. The values for the other men were essentially the same as those at S8. Five additional men studied at S24 showed the same decrease in 17-ketosteroid excretion as the preceding group.

During the rehabilitation period when the

[6] Miller, E. v. O., Mickelsen, O., and Keys, A., to be published.

[7] Miller, E. v. O., Mickelsen, O., and Keys, A., *Fed. Proc.*, 1947, **6**, 279.

TABLE II.

The Urinary Excretion of 17-Ketosteroids During Acute Starvation. All values for ketosteroid excretion are expressed as mg of androsterone per 24 hours. The mean urine volumes as cc per 24 hours are given. The standard deviations are given with the means. The decrease in ketosteroid excretion observed on the fourth day of starvation is expressed as a percentage of the control value (% Max. Decr.)

Subject	Control period	3rd day starvn.	4th day starvn.	% Max. Decr.
B	11.7	5.2	4.7	59.8
A	12.4	5.7	2.9	76.6
M	11.3	4.1	3.4	69.9
D	9.1	4.3	3.0	67.0
Pa	9.9	3.6	2.7	72.7
Pe	13.8	4.9	3.7	73.2
C	10.9	3.8	3.2	70.6
H	12.9	4.7	4.7	63.6
E	12.8	5.2	4.3	66.4
W	9.3	2.7	2.3	75.3
S	9.6	3.3	2.6	72.9
R	13.3	4.6	5.5	58.6
Mean	11.41 ± 1.66	4.34 ± 0.88	3.58 ± 1.00	68.9
Urine vols.	902 ± 245	745 ± 258	813 ± 445	

food intake was raised, the ketosteroid excretion increased. By R6, the mean excretion level for the 8 men examined then was 11.9 mg. The ketosteroid excretion returned practically to normal in spite of the fact that in even those subjects receiving the largest amount of food, body weight restoration amounted to a maximum of 18% of that lost during starvation with the values for most of the men below 7%. The recovery of physical fitness at this time was also very low. A subsequent year on an unlimited diet produced only a negligible increase in the 17-ketosteroid excretion. Subject 130 whose excretion was below normal at R6 was the one whose food intake during this period was the lowest. His recovery, on the basis of such factors as restoration of body weight, improvement of physical performance, etc., was considerably less than that of the other subjects. This subject was unusual in that his excretion level at R6 was so very much lower than the value at S24. In 2 cases (1 and 26) the excretion at R6 was much greater than at R57. There is no apparent explanation for these findings.

Results—acute starvation experiment. The 3 days of acute starvation produced a marked reduction in the 17-ketosteroid excretion of all 12 subjects (Table II). The average excretion during the control period was 11.41± 1.66 mg per 24 hours whereas that for the third day of the experiment was 4.34±0.88 and that for the fourth day was 3.58±1.00. In practically every case, the 24-hour urinary excretion was below the lower normal limits. On the fourth day of starvation, the ketosteroid output was still lower than that of the preceding day in all but 2 cases.

By far the greatest part of the reduction in the urinary excretion occurred by the third day of starvation. The decrease by that time averaged 62% of the control values whereas the total reduction was 69%. The reduction of the ketosteroid excretion ranged from 59 to 77%. There was no relation between the magnitude of the reduction and the control ketosteroid level.

The rate of excretion of ketosteroids in these two types of starvation was not influenced by the urine volume. In the semi-starvation experiment during the entire starvation phase the daily urine volume was about twice the control value. In the acute starvation experiment, on the other hand, the urine volume was markedly reduced during the starvation period.

Discussion. Both acute and semi-starvation with hard work produced marked reductions in the rate of total, neutral 17-ketosteroid excretion, the influence of acute starvation being much greater than that of semi-starvation. Any attempt to explain this change must involve a consideration of the functions influenced by each of the experiments. The plane of metabolism cannot be

the primary factor since in the acute starvation subjects this was at a very high level whereas in the semi-starvation subjects it was sharply reduced.

There is the possibility that the decrease in the ketosteroid excretion may be explained on the basis of a change in the activity of the testes. During the semi-starvation experiment there was a reduction in sexual functions as revealed by alteration in libido and in the morphology and physiological responses of the sperm collected at that time. Similar studies were not made in the acute starvation experiment, but here, too, at least libido was decreased. Other factors than the testes may be considered in explaining the decrease in ketosteroid excretion observed in acute and semi-starvation. This is apparent when one considers the marked differences in the ketosteroid excretion in these two experiments. Changes in the function and activity of the adrenals under these conditions can only be surmised at the present time.

The hard physical work in the acute starvation experiment had no influence on the ketosteroid excretion since the same work was done in the control period. Furthermore, in other experiments where the same work was done while the men were in caloric balance, there was no change in the ketosteroid excretion. Besides the physical "stress" in the acute starvation, both experiments were associated with considerable mental "stress" but in spite of this the ketosteroid excretion in each experiment decreased. This is contrary to other reports in the literature which have emphasized the increased ketosteroid excretion in "stressful" situations.[8]

Summary. The urinary excretion of total, neutral 17-ketosteroids was determined in 10 young men who, over a 6-months period, lost 24% of their original body weight. The reduction in ketosteroid excretion was 30% of the control values.

Twelve young men performed hard physical work during 4 days when they received no food. By the fourth day the urinary excretion of 17-ketosteroids was only 31% of the control value. The ketosteroid excretion was not influenced by the hard work since the subjects were trained to it before the start of the fast.

8 Pincus, G., and Hoagland, H., *J. Aviation Med.*, 1943, **14**, 173; Hoagland, H., *Science*, 1944, **100**, 63; Pincus, G., in *Recent Progress in Hormone Research*, Acad. Press, Inc., New York, 1947.

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Isolation of Toxoplasma from Cerebrospinal Fluid of a Living Infant in Holland.

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(Introduced by Albert B. Sabin.)

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Recent observations raise the supposition that toxoplasmosis is not infrequent in Europe. In most of the cases, the parasitological confirmation of the clinical diagnosis has been made post mortem by the demonstration of toxoplasma in brain sections. Toxoplasma have been demonstrated rarely in the cerebrospinal fluid during life of the patient by microscopic examination and animal inoculation. In the summer of 1947, however, we succeeded in this.

Our patient, the second child of apparently healthy parents, was a 6-weeks-old boy who did not show any signs of illness during the first few weeks of life. On August 7, the infant was admitted to the hospital, showing

TABLE I.
Toxoplasma Neutralization Tests in Rabbits.

Rabbit No.	Toxoplasma strain	Toxoplasma mixed with	Dilution of mouse brain suspension 1:10	1:50	1:500	1:5000
203	Bk	Tyrode's sol.	++++	+++	+	—
	Bk	Mother's serum	+	+	—	—
239	S	Tyrode's sol.	++	+	+	—
	S	Mother's serum	+	—	—	—
232	Bk	Tyrode's sol.	++++	++++	++	—
	Bk	Immune monkey serum	++	+	—	—
	S	Tyrode's sol.	++++	++++	+	—
	S	Immune monkey serum	++	+	—	—

++++ Strong swelling and necrosis.
+++ Strong swelling without necrosis.
++ Moderate swelling without necrosis.
+ Slight swelling.
— No reaction.

TABLE II.
Cross Immunity Between "Bk" and "S" Strains of Toxoplasma.

Rabbit No.	First inoculation Date	Strain	Reaction	Second inoculation Date	Strain	Reaction	Strain	Reaction
250	Oct. 14	S	++++	Dec. 1	S	+	Bk	+
202	Nov. 12	S	++++	1	S	++	Bk	++
213	7	Bk	++++	1	S	++	Bk	+
214	7	Bk	++++	1	S	++	Bk	++

Significance of + to ++++ as in Table I.

twitchings of arms and legs, rotations of the eye-balls, developing hydrocephalus, neck stiffness, opisthotonos, muscle rigidity and scissor stand of the legs; soon convulsions appeared.

Dr. C. D. Binkhorst, who examined the eyes, diagnosed anterior chorioretinitis, bilateral peripheral ablatio retinae, atrophy of the papillae and strabismus convergens. Dr. Berkyens, who performed a ventriculography, reported displacement and enlargement of the lateral ventricles, especially of the right one which was displaced to the left, ½ inch beyond the median line.

The cerebrospinal fluid, obtained by ventricular puncture, contained xanthochrome and tryptophane. The reactions of Nonne and Pandy were strongly positive, and there were 196 cells per cu mm. In the smear of the centrifuged sediment of this fluid (August 7) we found many halfmoon-shaped parasites resembling toxoplasma. They were not found in the blood, urine and bone-marrow.

On August 11, fresh cerebrospinal fluid was inoculated intracerebrally into 10 mice; 8 of them died after 7 to 25 (average 15) days, and toxoplasma could be demonstrated in the brains. In serial transmissions, the length of the incubation period decreased and from the third intracerebral passage 100% of the mice died after 5 to 6 days.

Guinea pigs inoculated intracerebrally with fresh cerebrospinal fluid from the patient, remained healthy, but those inoculated with toxoplasma-containing mouse brain suspensions died after 12 to 15 days.

Dr. A. B. Sabin kindly provided us with a toxoplasma strain of human origin (called by us strain S) for comparison with ours (strain Bk). We carried out a number of neutralization experiments (rabbit skin tests as described by Sabin[1]) with both strains, which were tested against serum of the infant's mother and against lyophilized toxoplasma immune monkey serum. The latter

[1] Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 6.

we also received by courtesy of Dr. Sabin. The results of the rabbit skin tests are recorded in Table I, from which it becomes evident that both of the sera neutralized the strains equally (between 10 and 50 skin test doses). The same was true with sera from a group of children suspected of having toxoplasmosis and with sera from their mothers; both of the strains were neutralized to the same titer.

Of 16 rabbits used for the skin test, 8 died 9 to 12 days after the inoculation. At autopsy an enlarged spleen with white foci, containing toxoplasma, was found regularly. Of the 8 surviving animals 4 were re-inoculated intracutaneously with 0.2 cc of a 10% mouse brain suspension at 4 separate spots, in order to test cross-immunity. Skin lesions of moderate size developed, but without necrosis. The results of the cross-immunity experiments are shown in Table II.

There was no striking difference in the skin reactions produced by the strains used. Only one of the animals died, but at autopsy no lesions specific for toxoplasmosis could be found, so that an intercurrent death is suspected. Evidence of cross-immunity has been given by this experiment.

As no differences in behavior, morphology, virulence, cross-neutralization and cross-immunity between the strains Bk and S could be observed, we believe that they are closely related or even may be identical.

The infant died on September 21. Treatment with sulphathiazole was not successful. At autopsy Dr. H. E. Schornagel found toxoplasma-containing mononuclear infiltrations in the meninges, the walls of the ventricles and the cerebral tissue, and foci of necrosis with many mononuclear cells and extracellular parasites. In the subcutaneous tissue, the psoas muscle and the diaphragma, some toxoplasma-containing mononuclear infiltrations were found.

Summary. This is a report of a case of toxoplasmosis occurring in Holland in a six-week-old infant, in whom during life toxoplasma could be shown in the cerebrospinal fluid by smear and also isolated by mouse inoculation. The serum of the infant's mother showed a strongly positive neutralization reaction, which was carried out by Sabin's rabbit skin test. In cross-neutralization tests and cross-immunity experiments on rabbits, the Dutch strain appeared to be closely related, perhaps identical with an American one.

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Diffusion Constants of the *E. coli* Bacteriophages.

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For determining the diffusion constants of substances which can be obtained pure in relatively large amounts, the conventional optical method of Lamm is most suitable. Unstable substances like bacteriophages and animal viruses, however, are often irreversibly altered by the purification procedures, and diffusion measurements on such systems are then subject to large errors. The diffusion of such substances is better investigated using impure solutions that are quantitatively evaluated through measurements of their biological activities.

Hershey, Kimura and Bronfenbrenner[1] have estimated the relative sizes of T^1 and T^2

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[1] Hershey, A. D., Kimura, Frances, and Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 7.

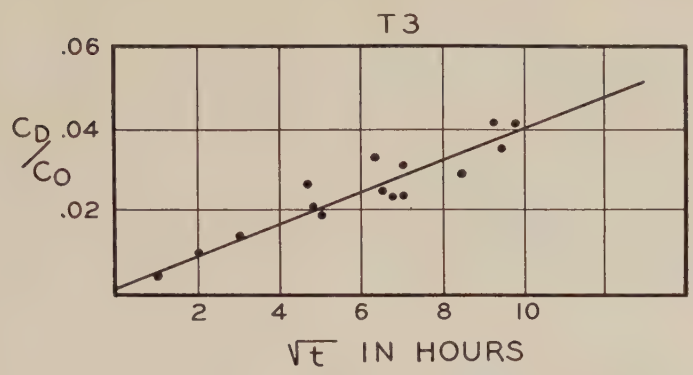

FIG. 1.
Ratio C_D/C_O plotted against the $\sqrt{t}$ for T_3 bacteriophage.

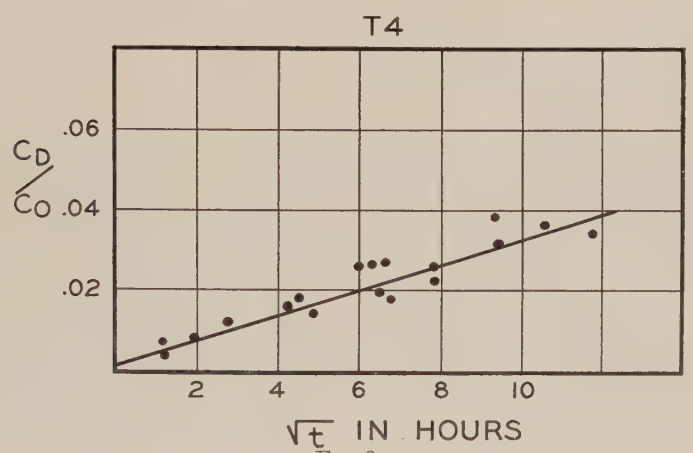

FIG. 2.
Ratio C_D/C_O plotted against the $\sqrt{t}$ for T_4 bacteriophage.

bacteriophages against *E. coli* by measuring their diffusion through thin agar, but such measurements are difficult to evaluate because of the different adsorption of these bacteriophages by the pores of the agar. The present paper is a preliminary account of the application of another diffusion technique to a tailed, T^4, and a tailless, T^3, bacteriophage. These bacteriophages were chosen to find out if the tail influences diffusion as well as to compare particle sizes computed from diffusion measurements with those measured with the electron microscope.

The method is essentially that previously employed by the author to determine the diffusion constant of African horse sickness virus.[2,3] The apparatus consists of 4 multi-chambered diffusion cells clamped onto a base plate provided with levelling screws. Sharp interfaces between bacteriophage and medium were formed by filling adjacent cells with medium and bacteriophage suspension and by rotating the top sections of the cells relative to the bottom until the 2 liquids came into exact apposition to one another. This was done by watching the index lines on the sides of the cells. The bacteriophage solution was diluted with 1% glucose broth solution to increase boundary stability and to insure a diffusion process free from disturbances by convection. This glucose broth solution, due to its higher density than the medium above it, forms a sharp boundary in the cell; the high rate of diffusion of its glucose into the broth produces a density gradient in the chamber into which the phage diffuses.

After suitable intervals the virus that dif-

[2] Polson, A., *Nature*, 1944, **154**, 823.

[3] Polson, A., forthcoming publication in *The Onderstepoort Journal.*

TABLE I.

Phage	$D \times 10^7$ cm²/sec (obs.)	η_m	T	$D_{20} \times 10^7$ cm²/sec (corr.)	Particle diameter in mμ
T_3	1.19	0.0101	295	1.19	36.2
T_4	0.798	0.0101	295	0.798	55.0*

* This figure is an equivalent spherical diameter, since this phage T_4, due to its tail, is obviously non-spherical.

fused past the initial boundary was isolated from that in the bottom by rotating the top sections to their cut-off position. The phage contents of the diffusates as well as of the original material in the lowest section were then determined by the usual plaque-count method.

The results are given in graphical form in Fig. 1 and 2, where the ratios of concentration in the diffusates C_D to that in the original material C_O are plotted against the square root of t, the time in hours. It is clear from these figures that C_D/C_O is a linear function of $\sqrt{t}$, a necessary requirement from the laws of diffusion in solutions.

Average diffusion constants were calculated from these data using the equation:[3]

$$D = (C_D/C_O)^2 \cdot \frac{H^2 \pi}{t}$$

where t is the time of diffusion in seconds, and H is the height of the column of liquid above the original boundary, regarding the chambers in the diffusion cells as cylinders. The value of H in these experiments has been 3 cm. Diffusion constants at the temperature of the experiment (22°C) calculated in this way are given in the second column of Table I. Constants for 20°C have been calculated from these with the expression:

$$D_{20°} = D_{22°} \cdot \frac{293°K}{295°K} \cdot \frac{\eta_m}{\eta_w}$$

where D_{20_o} is the diffusion constant at 20°C, η_m is the viscosity of the medium, and η_w is the viscosity of water at 20°C.

Particle sizes were computed from the well-known Einstein equation for the diffusion of a spherical particle:

$$D = \frac{RT}{N} \cdot \frac{1}{6 \pi r \eta}$$

where R is the gas constant, T is the absolute temperature, N is Avogadro's number, r is the radius of the particle, and η is the viscosity of the medium. This yields the diameters of the last column of the table. They are to be compared with the diameters of T_3, 45 mμ, and of T_4, 65 x 80 mμ, as measured from previous electron micrographs.[4]

It is clear that the tail of the bacteriophage T_4 does not contribute to its motion in a liquid medium. The slightest propulsion that such a tail might give the particle would have increased its diffusion constant tremendously since diffusion increases as the square of the distance moved in Brownian motion.

[4] Delbrück, M., *Biol. Rev.*, 1946, **21**, 30.

16281

A Free Swing Writer for Recording With Very Light Pressure on a Smoked Surface.

ALBERT E. AFFORD. (Introduced by S. Goldschmidt.)

From the Department of Physiology, School of Medicine, University of Pennsylvania.

The writing device to be described was developed for use in situations where recording on a smoked surface was unsatisfactory with the conventional type of "fixed" writing

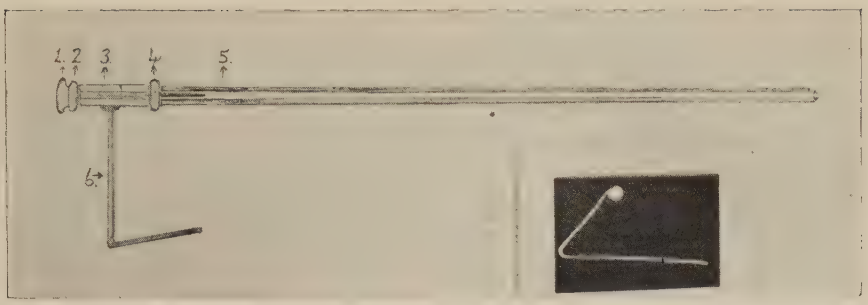

FIG. 1.

The writing device (side view) attached to reed. 1. Pin—length 2 cm. 2. Collar (glass bead), bore 1 mm, O.D. 2 mm. 3. Roller bearing (glass bead), bore 1 mm, O.D. 2 mm, length 6 mm. 4. Collar (glass bead), bore 1 mm, O.D. 2 mm. 5. Straw reed, bore 1 mm, O.D. 2 mm, 8 cm long. 6. Writing point—aluminum wire—gauge 24, 8 cm long. Attached to bearing with sealing wax. Approximate weight 200 mg. The insert shows an end view photograph of the writer.

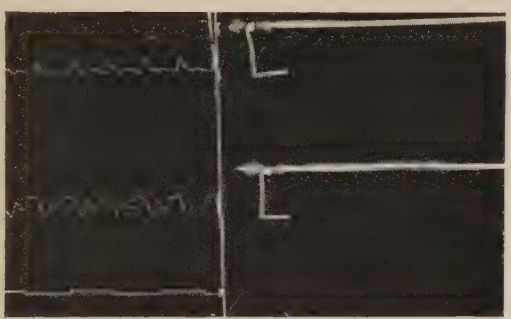

FIG. 2.

Actual tracings and arrangement for recording simultaneously the jugular pulse and the brachial artery utilizing the "free swing" writing lever attached to a tambour (not shown).

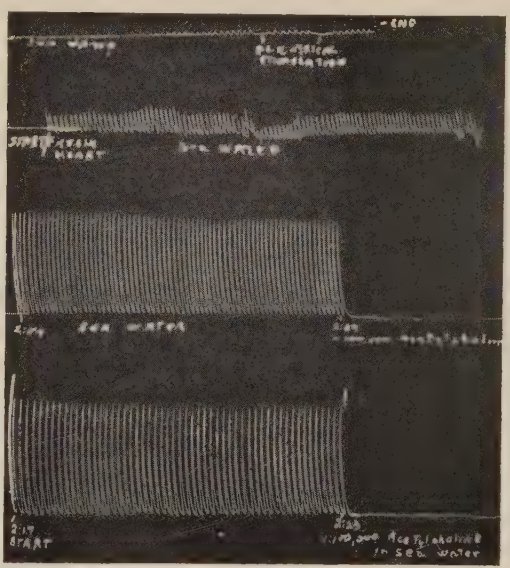

FIG. 3.

Kymograph tracings of excised heart of a clam (*Venus mercenaria*) beating in sea water, utilizing the "free swing" writer attached to light muscle lever.

point, because of frictional resistance. It has proved to be eminently more satisfactory, in the hands of students, than any device heretofore used. This is especially true in the recording of arterial and jugular pulse tracings (Fig. 2) and esophageal, gastric and duodenal movements in man. It alone has given acceptable tracings of the movements of an excised heart of a clam (venus mercenaria) (Fig. 3). The device (Fig. 1) is exceedingly simple and can be assembled in any laboratory. The writer is a short length (4 cm) of aluminum wire (24 gauge) bent to form an angle of approximately 60° in a plane at right angles to its point of suspension. The angle of the wire forms the writing surface. The pressure of the writer on the kymograph paper may be varied by altering the angle and the length of the free end of the aluminum wire; also by placing small bits of plasticine on the free end of the wire. The writer is attached with deKotinsky cement or sealing wax to a short length (6 mm) of glass tubular bead (1 mm bore), smoothed at the ends, which fits and moves freely over a brass-plated pin about 2 cm

long. This pin serves as a shaft or axle for the glass bearing to which the writer is cemented. Very smooth glass beads (1 mm bore, 2 mm long) on either end of the glass bearing hold it in place and prevent side motion. The pin is inserted into the end of a reed (1 mm bore, 8 cm long) such as is ordinarily used in kymograph recording. This reed is attached as usual to the recording tambour. Made and suspended as described above, the writer is free swinging and adapts itself to an uneven surface with uniform and minimal frictional resistance.

Summary. A free swinging writer which records on smoked surfaces with a minimum of frictional resistance is described; its construction is explained and illustrated. Specimen tracings of difficult recordings are illustrated.

16282 P

On New Adrenolytic Compounds.*

G. R. DE VLEESCHHOUWER. (Introduced by C. Heymans.)

From the J. F. Heymans Institute of Pharmacology, University of Ghent, Belgium.

In previous papers[1] we have demonstrated that several so-called sympathicolytic substances have mainly adrenolytic properties. In fact, these substances (benzodioxanes, dibenamine, dihydroergotamine, corynanthine) reverse the epinephrine-hypertension, but do not suppress sympathetic-vasopressor effects.

The present experiments were undertaken with two new synthetic compounds: α-naphtylmethylethyl-β-bromoethylamine HBr (SY-28) and β-(2-biphenyloxyethyl)-n-butyl-β-chloroethylamine HCl (SY-30).†

Some pharmacological properties of these compounds were studied by Loew, Achenbach and Micetich.[2,3,4]

Methods. The experiments were performed on chloralosed dogs. The blood pressure was recorded from a femoral artery. All drugs were injected intravenously and sometimes intra-arterially.

Results. A./α-naphtylmethylethyl-β-bromoethylamine HBr (SY-28).

1. *Effects on blood pressure, heart rate and respiration.* Intravenous injection of 1 mg/kg SY-28 produces an instant, moderate, transient hypotension and no change of heart-rate. The respiration becomes slow and irregular during the injection, but after a few minutes the dog is breathing normally.

Intra-arterial injection of small quantities of SY-28 (the animal being prepared according to the 3-manometers-method[5]) induces a marked and local vasodilatation.

Some hours after intravenous injection of SY-28, one remarks a slow and progressive fall of blood pressure. This corroborates Loew's observations, but we believe this hypotension to be due to a central depressing action of the drug.

2. *Effects upon neuro-vasomotor reactions.* The vasopressor and vasodepressor reflexes of carotid sinus origin are slightly depressed after injection of 1 mg/kg SY-28, while vasopressor effects of epinephrine are completely reversed. Although reversal of carotid sinus vasopressor reflexes never appears, a slow progressive depression of these reflexes has

* Aided by grants of the Ella Sachs Plotz Foundation, New York.

† Kindly supplied by Dr. Earl Loew and Parke, Davis & Co.

1 De Vleeschhouwer, G. R., *C. R. Soc. Biol.*, 1933, **115**, 187; 1934, **115**, 1247; 1935, **118**, 792; *Arch. int. Pharmacodyn. et Thér.*, 1935, **50**, 251; PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 151; in press.

2 Loew, Earl R., and Micetich, A., *Fed. Proc.*, 1947, **6**, 351.

3 Achenbach, P., and Loew, Earl R., *Fed. Proc.*, 1947, **6**, 304.

4 Loew, Earl R., personal communication.

5 Nolf, P., *Bull. Acad. Roy. Belg.*, 1902, p. 895.

been observed.

The neuro-vasopressor reflex originated by stimulation of the central end of the vagus is scarcely altered in the dog pretreated with SY-28.

The diphasic hypertension following electric stimulation of the splanchnic nerve, in normal dogs, is dissociated into a monophasic rise and a secondary fall of blood pressure after injection of 1 mg/kg SY-28. No reversal of the initial neuro-vasopressor response of sympathetic origin occurs.

3. *Effects on hypertensive substances*

Intravenous injection of 1 mg/kg SY-28 reverses the vasoconstrictor effects of epinephrine and inhibits its tachycardic action.

Same doses reduce or abolish the vasopressor effect of nicotine, ephedrine and veritol (β-p-oxyphenyl-isopropylmethylamine), but never induce a vasodilator response.

The hypertensive action of acetylcholine, in the atropinized dog, persists after injection of SY-28.

The vasopressor action of levo-arterenol‡ is notably depressed, at a time when neuro-vasopressor effects of sympathetic origin are nearly normal and epinephrine reversed by SY-28.

B./ β-(2-biphenyloxyethyl)-n-butyl-β-chloroethylamine HCl (SY-30).

According to Loew's and our observations, the pharmacological actions of SY-30 and SY-28 are similar, the SY-30 being less active.

Intravenous injection of 5 to 10 mg/kg has no immediate effect on blood pressure and heart rate, whereas intra-arterial injection produces a marked local vasodilatation, often preceded by a slight vasoconstriction. Later on the general blood pressure decreases progressively.

Vasopressor effects of carotid sinus origin are rapidly depressed but not reversed. Similarly, the neuro-vasopressor responses due to stimulation of the splanchnic nerve or other sympathetic nerves, may be diminished, but are never reversed after injection of SY-30.

While epinephrine hypertension is reversed and its tachycardic action completely abolished, the vasopressor activity of ephedrine, nicotine, dl-arterenol, acetylcholine (after atropinization) is weakened or blocked.

Finally, SY-30 paralyzes the cario-accelerator nerves.

Summary. The experiments showed that α-naphtylmethylethyl-β-bromoethylamine (SY-28) and β-(2-biphenyloxyethyl)-n-butyl-β-chloroethylamine (SY-30) are very active adrenolytic agents, with a weak sympathicolytic effect. The present and previous experimental observations once more demonstrate a marked dissociation between the vascular action of epinephrine and the neuro-vascular sympathetic transmission of excitations.

‡ Kindly provided by M. L. Tainter (*Science*, 1948, **107**, 39).

16283 P

Effect of Muscle Work Upon Level of Blood Glucose in the Eviscerated Rat.

DWIGHT J. INGLE.

From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

These experiments show that faradic stimulation of the gastrocnemius muscle accelerates the rate of fall of blood glucose in eviscerated and eviscerated-nephrectomized rats.

Methods. Male rats of the Sprague-Dawley strain were fed Friskies Dog Cubes. At a weight of 185 to 205 g, the inferior vena cava was ligated between the liver and kidneys to cause the development of a collateral circulation. Asepsis was preserved. When the animals reached 265 to 310 g they were anesthetized (cyclopal sodium) and eviscer-

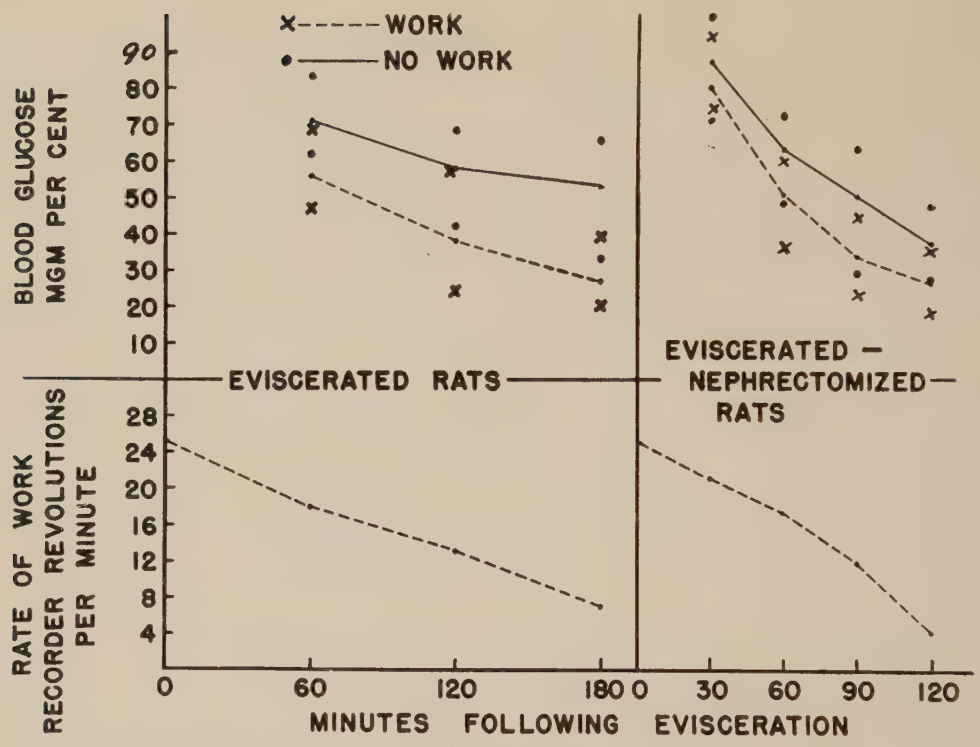

FIG. 1.

The effect of work upon the level of blood glucose in eviscerated and eviscerated-nephrectomized rats. Averages and range. Ten pairs of rats were used in each experiment.

ated by the method of Ingle and Griffith.[1]

In Exp. 2 the kidneys were removed at the time of evisceration. Immediately following the operation the animals were prepared for the stimulation of the gastrocnemius muscle to lift 100 g 3 times per second, according to the procedure of Ingle.[2] The animals were closely matched into pairs on the basis of body weight. One animal of each pair was subjected to work, and the second was kept without the weighting or stimulation of the muscle. Glucose was determined on tail blood by the method of Miller and Van Slyke.[3]

Experiments and Results. Ten pairs of rats were used in Exp. 1. One rat of each pair was subjected to stimulation of the gastrocnemius muscle for 180 minutes. Samples of blood were taken from each animal at the end of 60, 120 and 180 minutes following evisceration.

Ten pairs of eviscerated-nephrectomized rats were used in Exp. 2. One rat of each pair was subjected to the stimulation of muscle for 120 minutes. Samples of blood were taken 30, 60, 90 and 120 minutes following evisceration.

The data are summarized in Fig. 1. The rates of work and levels of blood glucose decreased more rapidly in the eviscerated-nephrectomized rats than in the eviscerated rats. In both series of animals the fall in blood glucose was accelerated by the stimulation of muscle. All of the "work" series developed convulsions and fatal hypoglycemia before convulsions were shown by any of the "no work" series.

Comment. The effect of nephrectomy in hastening the onset of hypoglycemia in the rat was previously known.[4] Although the

[1] Ingle, D. J., and Griffith, J. Q., Chapter 16, *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

[2] Ingle, D. J., *Endocrinology*, 1944, **34**, 191.

[3] Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

eviscerated rat lacks a pancreas and its hormone, insulin, it is capable of utilizing some glucose, as is readily shown when the organs responsible for the endogenous formation of glucose, *i.e.*, the liver and kidneys, are absent. The rate at which such animal removes glucose from the blood is further accelerated by muscular work. We propose to study the effect of muscle work upon the glucose load which the eviscerated rat can tolerate in the presence and absence of insulin.

Summary. In eviscerated and in eviscerated-nephrectomized rats stimulation of the gastrocnemius muscle accelerates the fall of blood glucose and shortens the time of survival.

4 Reinecke, R. M., *Am. J. Physiol.*, 1942, **136**, 167.

16284 P

Enhancing Effect of Mg^{++} on Clotting Activity of Ca^{++}

FRANK MALTANER.

From the Division of Laboratories and Research, New York State Department of Health, Albany.

Recent studies[1] on the activating effect of metallic salts on the hemolytic function of complement led to the conclusion that free bivalent cations were essential to the process and that the importance of Mg^{++} was more decisive than Ca^{++} or other cations studied.

In previous publications we have reported experiments indicating a close correlation between cephalin and Ca^{++} and their role in the coagulative and complementary activities of blood plasma or serum.[2-4] Moreover, in the clotting process the action of ionized calcium salts has been shown to be highly specific in that salts of even closely related elements, strontium and barium, had relatively little effect.

It seemed of importance therefore to determine whether magnesium, although inactive alone, might enhance the coagulative activity of calcium for plasma or serum.

The technic used in these experiments was similar to that described previously[3] except for the use of guinea pig plasma instead of rabbit plasma. Two preparations were employed: a 0.1% cell-free oxalated plasma for determining the direct coagulating effect of Ca^{++} or other ions and a more strongly oxalated and diluted plasma, the dioxalated plasma of Bordet and Delange[5] which is more resistant to clotting by direct recalcification and provides a satisfactory reagent for the detection of "thrombin" activity.

Oxalated plasma was obtained by bleeding from the carotid artery over paraffined surfaces into 1% sodium oxalate containing 0.5% NaCl, 1 part to 9 parts of blood, and removal of cells and platelets by centrifugation at 2°-6°C.

Dioxalated plasma was prepared by mixing 1 volume of cell-free oxalated plasma with 4 volumes of 0.2% sodium oxalate containing 0.85%NaCl.

The solutions of $MgCl_2$ and $CaCl_2$ were prepared by diluting molar solutions with 0.85% saline.

The cephalin was a 0.01% solution of phosphatidyl serine in 0.85% saline.

In the experiments of Fig. 1, 0.1 ml amounts of the molar concentrations of $CaCl_2$ or $MgCl_2$ indicated were used, the volume was made up to 0.6 ml with saline and 0.1 ml of

1 Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

2 Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1936, **30**, 417.

3 Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1937, **33**, 297.

4 Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *Am. J. Physiol.*, 1937, **119**, 80.

5 Bordet, J., and Delange, L., *Ann. de l'Institut Pasteur*, 1912, **26**, 657.

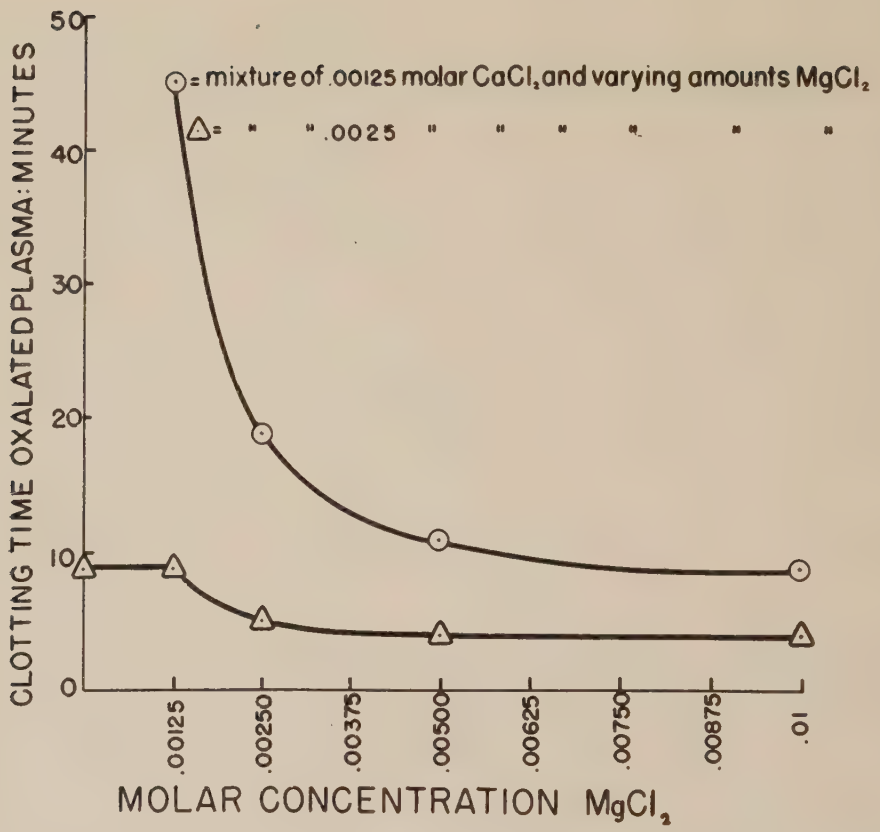

FIG. 1.
The enhancing effect of $MgCl_2$ on the coagulation of recalcified oxalated guinea pig plasma. No clotting was observed with any concentration of $MgCl_2$ without $CaCl_2$ during two hours' observation. 0.00125 molar $CaCl_2$ alone failed to clot within 2 hours.

oxalated plasma was added. Thus the effective molar concentration was one-seventh of that indicated in the figure. The tubes were placed in a 37°C water bath and clotting times determined.

In the experiments of Fig. 2 the quantities of guinea pig complement indicated were pipetted from 1:10 and 1:100 dilutions in saline, 0.1 ml of 0.00125 molar $CaCl_2$ and 0.1, 0.2, 0.3, or 0.4 ml of 0.00125 molar $MgCl_2$ were then added, and finally 0.1 ml of a 0.01% solution of phosphatidyl serine. The volume was brought to 0.6 ml with saline. The effective molar concentration of $CaCl_2$ or $MgCl_2$ was thus one-sixth of that indicated in the figure. Mixtures were incubated 6 minutes in a 37°C water bath, 0.1 ml of the oxalated and diluted plasma was then added, and the clotting time determined. In the absence of complement the indicated amounts of other reagents, either alone or in combination, had no clotting action on dioxalated plasma nor did the complement alone have such activity.

The enhancing effect of $MgCl_2$ on the coagulation of recalcified cell-free oxalated guinea pig plasma is illustrated in Fig. 1.

The effect of $MgCl_2$ on the clotting activity of guinea pig serum (complement) resulting from preliminary incubation of the serum with cephalin and $CaCl_2$, the so-called prothrombin activation, is illustrated in Fig. 2. These results were obtained with minimum quantities of $CaCl_2$. Similar results were obtained when larger doses of $CaCl_2$ were used but the effect of $MgCl_2$ did not appear so great as the optimum dosage of $CaCl_2$ was approached.

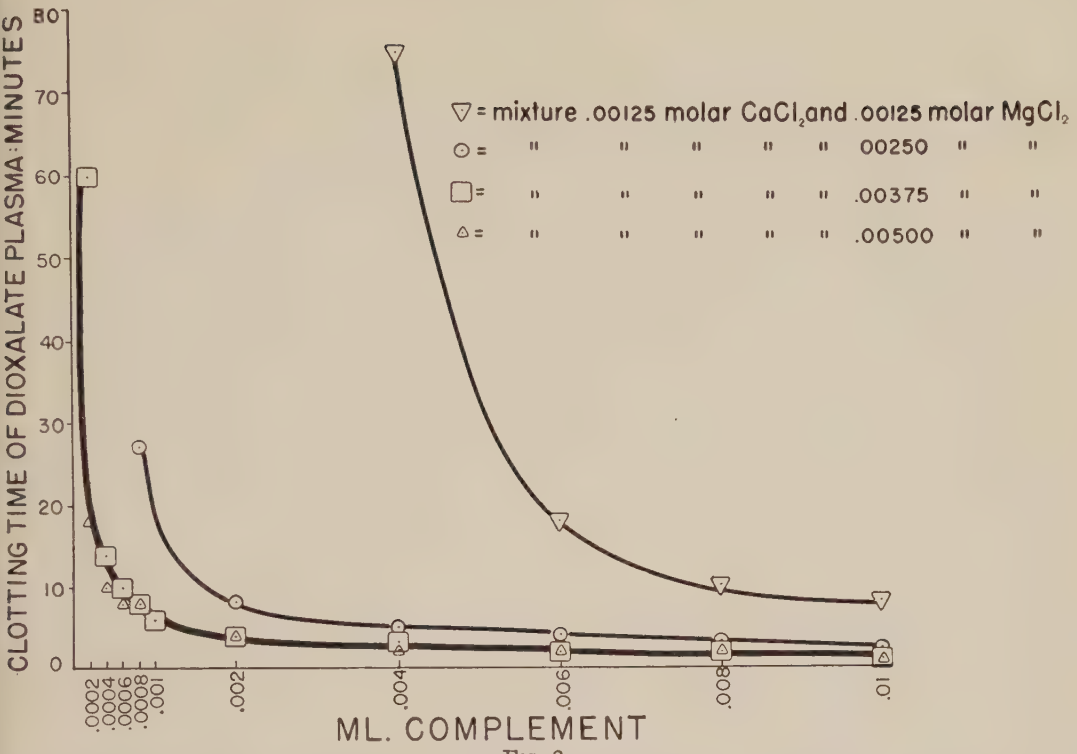

FIG. 2.

Enhancement of clotting activity of serum (complement). No clotting was observed with 0.00500 molar $MgCl_2$ without $CaCl_2$ during 2 hours' observation. 0.00125 molar $CaCl_2$ alone failed to clot within 2 hours.

Conclusions. The results of the experiments demonstrated that whereas ionized magnesium salts ($MgCl_2$) alone do not possess the coagulative activity of calcium salts, they do markedly enhance the effect of calcium. The enhancement is more apparent when the Ca ion concentration is relatively low and the concentration of magnesium salts exceeds that of calcium salts. Thus the action of Mg^{++} is secondary or accessory, the more essential and decisive role in clotting being played by Ca^{++}.

The parallelism between the enhancing effect of Mg^{++} in the coagulative and the complementary activities of the blood provides additional support for the close relationship of these two phenomena.

16285

Nutritional Requirements of *Trichomonas vaginalis.**

Alfred B. Kupferberg, Garth Johnson, and Herbert Sprince.

From the Division of Microbiology, Ortho Research Foundation, Raritan, N.J.

Very little is known about the growth factor requirements of *Trichomonas vaginalis.* In an introduction to the study of growth factor effects Johnson[1] observed that ascorbic acid, glutamic acid and choline had a stimulating effect upon cell multiplication, and that where nicotinamide, folic acid and approximately 10% human serum were employed liver infusion was satisfactorily replaced. Sprince and Kupferberg[2] reported a medium which could be used as a starting point for the development of a chemically defined culture fluid for the sustained growth of *Trichomonas vaginalis.* A highly complex medium was devised with numerous growth factors included. In these two studies materials of natural origin were added to the media in such concentrations as to mask the essentiality of added *known* growth factors. It is the purpose of the present study to demonstrate that by reducing the serum and trypticase content of the medium of Sprince and Kupferberg a requirement for pantothenic acid and for phosphate was established for *Trichomonas vaginalis.* It was thought that an effort to simplify the above culture medium might reveal a requirement for certain of the component growth factors. Furthermore, the need for a medium to be used for routine maintenance of cultures as well as for testing the antitrichomonas activity of compounds made a cheaper medium desirable. The present study reports the effects of withdrawal of the above growth accessory substances upon cell multiplication of bacteria-free *Trichomonas vaginalis.*

Methods. Strain No. 2 of bacteria-free *Trichomonas vaginalis* was used as the test organism. The basal medium consisted of the *basal* trypticase medium previously reported by Sprince and Kupferberg.[2] The inoculum was prepared by washing cells grown for 48 hours in the trypticase medium. This was accomplished by centrifuging and resuspending in sterile Ringer's solution 4 times. The population was not adjusted. The inoculum consisted of 0.03 ml of the washed suspension. Five tubes were employed for each experimental series except where otherwise indicated. The contents of the tubes were agitated with the tip of the pipette in order to disperse the serum and inoculum. Subcultures were made at 48 hour intervals. Populations were determined by hemocytometer count in the second transplant following the first culture. This medium contained 2 ingredients of natural origin, serum and trypticase. An effort was first made to determine the minimal amounts of serum required for sustained culture in order that sensitivity to withdrawal of the known vitamins might be determined.

Effect of reduction of serum concentration. In order to increase the accuracy of measurement the serum was first diluted to various concentrations in 100 ml of modified Ringer's solution containing 0.6% NaCl and 0.01% $NaHCO_3$, KCl and $CaCl_2$. The diluted serum was then added in 2 ml volumes after Seitz filtration. The effects of graded amounts of human serum upon cell multiplication are shown in Table I. Similar results were obtained in a duplicate series. Since the cell counts in cultures containing 0.05 ml of serum were more uniform than those in which the minimal 0.03 ml was used, it was decided to employ 0.05 ml in the experiments to follow.

* The technical assistance of Mrs. Mary Williams, Miss Ruth Grossman, and Mr. LeRoy Markle is hereby acknowledged.

We wish to thank the staff of the Division of Bacteriology and Serology of the State of New Jersey Department of Health, Trenton, for generous amounts of human blood serum.

1 Johnson, J. G., *J. Parasitol.*, 1947, **33**, 189.

2 Sprince, H., and Kupferberg, A. B., *J. Bact.*, 1947, **53**, 435.

TABLE I.
Effect of Graded Amounts of Serum on Cell Multiplication of *Trichomonas vaginalis.*

Ml undiluted serum per 10 ml final medium	Population in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
.01	0	0	0	0	0
.03	31	57	101	112	88
.05	470	410	240	450	440
.07	1310	1240	700	1190	760
.10	1910	1440	1860	2090	2190
1.0	2510	2470	2310	2270	2310

* Similar results were obtained in a duplicate series.

TABLE II.
Effect of Omission of Vitamins in Groups.

	Population of *Trichomonas vaginalis* in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
Control	350	190	280	360	280
" minus Group A	0	0	0	0	0
" " " B	390	460	190	400	400
" " " C	440	450	440	480	400

* Similar results were obtained in a duplicate series.

The effect of withdrawal of known growth factors. These materials were first deleted in groups. Where populations were affected by the withdrawal of a group of compounds, the effect of withdrawal of individual compounds was then explored.

Results. Preliminary studies showed that thiamine HCl, p-aminobenzoic acid, pyridoxine HCl, pyridoxamine HCl, pyridoxal HCl and inositol were not required for sustained growth in the presence of trypticase and serum. The withdrawal of sodium acetate, sodium bicarbonate, asparagine, ribose, adenine sulfate, guanine HCl, xanthine, and uracil was also without effect upon the growth of *Trichomonas vaginalis.* These materials were therefore deleted in the following study of essentiality of vitamins. In the experimental study upon withdrawal of 3 groups of vitamins, however, a specific vitamin requirement was uncovered. The compounds in this study were grouped as follows with the amount of each given in μg per 10 ml of final medium: A. Choline 80 μg, riboflavin 8.0 μg, calcium pantothenate 3.2 μg, nicotinic acid 3.2 μg; B. Biotin 0.8 μg, folic acid 0.8 μg; C. Ascorbic acid 1000 μg. The control medium contained groups A, B, and C plus trypticase 2% and serum 0.5%. Agar, maltose, cysteine HCl, and Ringer's were present in the same concentrations as in the original trypticase medium. These groups were then withdrawn from the medium separately. The results obtained are shown in Table II.

It is demonstrated in Table II that one or more vitamins in Group A are essential. Growth equal to that of the control was obtained where Groups B and C were omitted from the medium. The vitamins in Group A, namely: choline chloride, riboflavin, calcium pantothenate and nicotinic acid were then withdrawn singly. The effect of single omissions of these 4 vitamins may be noted in Table III. From these data we may conclude that pantothenic acid is an essential metabolite of *T. vaginalis.* In Table IV it is also shown that the effect of pantothenate is not dependent upon the presence of added vitamins.

It has been demonstrated by Denko *et al.*[3]

[3] Denko, W. D., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

TABLE III.
Effect of Omission of Single Vitamins.

Vitamins omitted from control	Population of *Trichomonas vaginalis* in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
None (control)†	710	570	540	950	720
Choline	620	620	940	550	660
Riboflavin	630	610	900	660	690
Nicotinic acid	630	880	540	670	870
Ca pantothenate	1	2	0	0	0

* Similar results were obtained in a duplicate series.
† Same as control in Table II except Groups B and C omitted.

TABLE IV.
Effect of Increase in Serum and Omission of All Known Vitamins.

Simplified medium†	Population of *Trichomonas vaginalis* in second transplant at 48 hr (cells/mm³)* Cultures				
	1	2	3	4	5
0.5% Serum	0	0	0	0	0
Ca Pant. and 0.5% Serum	780	530	640	620	580
Minus Ca Pant., plus 5% Serum	2150	1950	1890	1920	2040

* Similar results were obtained in a duplicate series.
† Unessential ingredients omitted from medium.

TABLE V.
Effect of Graded Amounts of Trypticase on Cell Multiplication of *Trichomonas vaginalis*.

% Trypticase in final medium	Population in second transplant at 48 hr (cells/mm³) Cultures			
	1	2	3	4
.008	10	9	7	14
.04	64	70	62	64
.08	151	108	111	165
.16	230	300	350	240
.24	450	420	410	420
.48	940	540	870	810
.72	1210	1300	1020	990
.96	1500	1600	1560	1500
1.20	1830	1640	1760	1620
1.36	1750	1810	1830	1460
1.60	1970	1750	1860	1680
2.00	2050	1920	1970	1750

that normal whole blood has on the average 33 μg of pantothenic acid per 100 ml. The effect of increased amounts of blood serum upon the requirement for added pantothenate was therefore explored. The medium was prepared minus the addition of all known vitamins and the serum content was increased from 0.5% to 5.0%. The results are indicated in Table IV.

As is shown in Table IV no need exists for Ca pantothenate when 5% human serum is employed in the medium in the presence of 2% trypticase (BBL).

Effect of reduction of trypticase concentration. Using the *basal* medium of Sprince and Kupferberg[2] the trypticase content was varied between 0.008 and 2.0% in the presence of 5% serum. The effect of these reductions is shown in Table V.

As can be seen from Table V, 0.24% of trypticase gave a uniform cell count. In several duplications of this experiment, trypticase concentrations below this value produced widely varying populations in the quadru-

TABLE VI.
Effect of Simultaneous Reduction of Serum and Trypticase Concentrations.

Conc. % final vol. Trypticase	Serum	Population cells per mm³ Culture 1	Transfer 1	Transfer 2	Transfer 3	Transfer 4
2	5	1980	2100	2300	1980	1870
2	0.5	680	700	610	690	690
0.24	5	560	440	480	440	470
0.24	0.5	0	0	0	0	0

TABLE VII.
Effect of Addition of Phosphate to Medium Containing Reduced Serum and Trypticase.

	Population cells per mm³ Culture 1	Transfer 1	Transfer 2	Transfer 3	Transfer 4
Serum 0.5%— Trypticase 2%	970	760	930	860	880
Serum 0.5%— Trypticase 0.24%	0	0	0	0	0
Serum 0.5%— Trypticase 0.24% KH_2PO_4 0.027%	276	304	315	260	270

plicate cultures with aberrations in shape and motility. For these reasons 0.24% trypticase was selected for use in further investigations.

Effect of simultaneous reduction of serum and trypticase concentrations. From the foregoing data independent reduction of serum to 0.5% and of trypticase to 0.24% is shown to produce low but consistent populations. The effect of simultaneous reduction of serum and trypticase to these low values is shown in Table VI.

The above data reveal that this medium which contains a full complement of added growth factors is deficient in some respects when serum is reduced to 0.5% and trypticase to 0.24%. It was then supplemented with a variety of mineral salts in order to determine whether this deficiency was due to a lack of an essential salt. Table VII shows the effect of the addition of phosphates.

The data in Table VII demonstrate a requirement for phosphate. Substitution of $NH_4H_2PO_4$ gave results comparable to those produced by the salts of potassium. The addition of $MgSO_4$, $FeSO_4$, $MnSO_4$ and $MnCl_2$ was without effect. The optimal concentration of phosphate was found at 0.027%. At this phosphate level the population of trichomonads was approximately 30% of that obtained with 2% trypticase.

Reduction of components in routine culture medium. From the data gathered in the foregoing study it is obvious that in a medium containing adequate serum and trypticase, added growth factors are not required. The trypticase also supplies sufficient phosphate. In view of this fact, the need for the salts included in the Ringer's solution was investigated. Deletion of the Ringer's solution produced no change in the ability of the medium to support optimal populations in serial cultures.

Composition and Population of STS medium. The elimination of all non-essential components left a Simplified Trypticase Serum medium having the following composition per 1000 ml of final medium: Trypticase (BBL) 20 g, cysteine HCl 1.5 g, maltose 1.0 g, Difco agar 1.0 g, distilled water to make 950 ml. The medium was adjusted to pH 6.0, with 1N HCl or 1N NaOH, heated in a boiling water bath until the agar was completely dissolved. The solution was filtered while hot through porous Reeve-Angel filter paper No. 845. To the warm filtered mixture was now added 0.6 ml of

0.5% methylene blue to serve as an indicator. The use of an indicator is optional. After being cooled to 46°C the mixture was readjusted, if necessary, to pH 6.0 using the Beckman pH meter. The solution was then brought back to 950 ml with distilled water, tubed in 9.5 ml volumes, autoclaved at 15 lb pressure for 15 minutes, and allowed to cool. To render the medium complete 0.5 ml of sterile, undiluted human serum was added to each tube giving a final volume of 10 ml.

Populations were counted through 11 serial cultures and compared with those in the original medium of Sprince and Kupferberg. It was found that the simplified medium supported populations equal to those in the original medium. Over 50 serial transfers have been maintained to date without visible differences in shape, size and degree of motility of the protozoa. Examination of living specimens by phase microscopy and stain techniques failed to show any difference in morphology. Three strains of *T. foetus*† and one strain of *T. gallinae* have been carried successfully in this medium at this laboratory. The former species requires adjustment of the medium to pH 7. It has recently been observed that all 3 species are satisfactorily supported by Baltimore Biological Laboratory Thioglycollate medium with dextrose and Eh indicator. This medium contained phytone and trypticase. It requires adjustment to pH 6 for *T. vaginalis* and *T. gallinae*. All 3 species require the addition of serum.

Conclusions. 1. Pantothenic acid has been demonstrated to be an essential metabolite for *Trichomonas vaginalis*. 2. A requirement for phosphate has been established. 3. A culture medium containing a greatly reduced number of components has been developed for routine use.

† Received from Dr. B. B. Morgan, University of Wisconsin.

16286

Turnover Rate of Phospholipid Phosphorus in the Liver of the White Rat.

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The purpose of the experiments reported here is to provide a quantitative estimate of the turnover rate of phospholipid phosphorus in the liver of the white rat under normal conditions of equilibrium, that is, while the actual concentration of the phospholipid phosphorus in the liver is not changing.

The concept of "turnover" is one of dynamic equilibrium in which, during an interval of time dt, a number of phosphorus atoms of mass dm' "enter" the hepatic phospholipid molecules, and the same number "leave," so that the net mass of phospholipid phosphorus remains unchanged. The rates concerned are defined as follows:

$$r = \frac{dm'}{dt} \qquad (1)$$

$$R = \frac{r}{m} = \frac{dm'}{mdt} \qquad (2)$$

where r is the mass turnover rate; R is the proportional turnover rate; dm' is the mass of phospholipid P which is "turned over" in time dt; m is the mass of phospholipid phosphorus.

The method used for calculation of the rates is based on the formulation of Zilversmit, Entenman and Fishler.[1] These authors

[1] Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, 1943, **26**, 325.

analyzed mathematically the changes of specific activity of a substance which is in equilibrium with a single precursor, the activity of which itself may be changing. Their analysis showed that the proportional turnover rate is equal to the rate of change of specific activity of substance at any instant, divided by the difference of the specific activity of precursor and substance at that instant. If one considers the changes occurring over a finite interval of time, instead of the instantaneous relations, the proportional turnover rate is equal to the change per unit time of activity of substance, divided by the difference of the *mean* specific activity of precursor and substance in the interval. So far as we know, this method has not previously been applied for the calculation of any actual turnover rate.

Changes of radioactivity of hepatic phospholipid have been studied in terms of relative activity, that is the ratio of specific activity of phospholipid phosphorus to that of hepatic or plasma inorganic phosphorus, or in relation to the activity of the phosphate administered.[2-4] These indexes, however, are not measures of the true proportional turnover rate, and will appear as changing values during the course of an experiment, or will vary according to the regimen of administration of radioactive material, even when the turnover rate itself is actually constant.

Hevesy and Hahn,[2] and Hahn and Tyren[5] and Hevesy[6] have suggested calculating the percentage renewal of phosphatides of the liver by comparing the activity of the phosphatide P at the end of the experiment with the average activity of the inorganic P during the experiment, under conditions when the specific activity of the inorganic P of the liver does not change much. They reported the percentage of newly formed phosphatides in the liver of the rat in 4 hours to be 20% with a strong variation from animal to animal. However, the specific activity of the inorganic phosphorus did in fact change considerably during the course of our experiments. Moreover, even if the hepatic inorganic phosphorus, considered as precursor, remains constant, it is still necessary in accordance with the formulation of Zilversmit and associates to subtract the mean activity of the phospholipid P itself.

The method used for calculating R is based on the assumption that the hepatic inorganic phosphate is the immediate and only precursor of phospholipid phosphorus or, more to the point, that its specific activity is not materially different from that of the immediate precursor. Evidence that this condition is fulfilled with sufficient closeness to yield a reliable estimate of R is given if the value of R, when calculated for successive intervals of the experiment, is reasonably stable and shows no time trend. Still more convincing evidence is provided if the calculated value is essentially the same, even if the shape of the activity time curves themselves is markedly altered by changing the rate of administration of the activated material. As will be seen later, both criteria were used in the experiments here reported, and both confirmed the values obtained.

Experimental methods. Adult male white rats weighing approximately 200 g maintained on the stock commercial diet of "friskies" were fasted for 20 hours prior to the administration of P^{32}, and during the subsequent interval before removal of specimens. The P^{32}, obtained from the Massachusetts Institute of Technology, was administered as the dibasic sodium phosphate. In the first series of experiments reported later each rat received by a single intravenous injection 12 microcuries of P^{32} and not more than 0.02 mg of P^{31}. In the second series the P^{32} was administered by continuous intraperitoneal injection at the rate of 3 microcuries per hour. At specified intervals after the first administration the rats were anesthetized with pentobarbital sodium. Blood was withdrawn by cardiac puncture into a heparinized syringe. The liver was quickly removed and frozen

2 Hevesy, G., and Hahn, L., *Kgl. Danske Videnskab. Selskab, Biol. Medd.*, 1940, **15**, 66.

3 Artom, Camillo, Sarzana, Gaetano, and Segré, Emilio, *Arch. Int. Physiol.*, 1938, **47**, 245.

4 Chaikoff, I. L., *Physiol. Rev.*, 1942, **22**, 291.

5 Hahn, L., and Tyren, H., *Arkiv f. Kemi, Mineral. och Geol.*, 1946, **21** A, No. 11.

6 Hevesy, G., *Arkiv f. Kemi, Mineral. och Geol.*, 1947, **24** A, No. 26.

TABLE I.
Values Obtained After a Single Intravenous Injection of 12 Microcuries of P^{32} and Not More Than 0.02 mg P^{31}.*

1	Hr after administration, t		0.5	1	2	4
2	Rats		7	7	10	21
3	Body wt, g		189 ± 4.9	188 ± 4.7	214 ± 6.3	202 ± 3.8
4	Liver wt, % of body wt		3.2 ± 0.2	3.5 ± 0.4	3.8 ± 0.2	3.3 ± 0.1
5	Mg P per 100	Plasma inorganic P	6.9 ± 0.4	6.8 ± 0.4	7.5 ± 0.4	7.9 ± 0.2
6	g liver	Hepatic " "	31.8 ± 2.1	30.2 ± 1.6	31.1 ± 1.3	33.0 ± 1.1
7		" lipid "	137 ± 3.0	139 ± 4.4	133 ± 5.7	137 ± 1.8
8	Specific	Plasma inorganic "	3,876 ± 203	2,015 ± 262	1,174 ± 87	744 ± 33
9	activity at	Hepatic " "	3,063 ± 280	2,578 ± 377	1,837 ± 154	1,102 ± 62
10	time t	" lipid "	64 ± 7	133 ± 14	245 ± 24	339 ± 18
11	Relative activity, hepatic lipid P: inorganic P		0.02	0.05	0.13	0.31
12	Mean specific	Hepatic inorganic P	2,618	2,641	2,410	1,932
13	activity to	" lipid "	30	67	126	211
14	time t	Diff.	2,588	2,574	2,284	1,721
15	Change of specific activity, lipid P per hr		128	133	122	85
16		R	0.049	0.052	0.053	0.049

* All quantities are means for the number of rats given in row 2; the values after the ± are the standard errors of the means. The mean specific activity to time t was obtained as explained in the text.

in a mixture of carbon dioxide ice and alcohol and then was pulverized between chilled steel blocks.

An aliquot of the liver was extracted with trichloracetic acid, with the usual precautions, and the inorganic phosphate was precipitated as the magnesium ammonium salt. Traces of some organic phosphates are included in this precipitate but the amount and the radioactivity of these do not materially affect the value obtained for either the amount or the specific activity. The phosphorus content was determined by the method of Fiske and Subbarow[7] and the radioactivity was measured with a Geiger-Muller counter of the immersion type.[8] Specific activity is reported as counts per second per mg P after correction for decay from the time of injection to the time of counting. Direct measurements of the inorganic phosphate in a trichloracetic acid extract of the plasma were made without the preliminary precipitation.

Approximately 3 g of pulverized frozen liver were ground immediately with sufficient anhydrous Na_2SO_4 to remove the water.[9,10] The tissue was then extracted overnight at room temperature with 50 ml of alcohol-ether. The extract was filtered, the residue was washed once with alcohol-ether and twice with ether and the volume was made up to 100 ml. This procedure in the presence of plasma or tissue protein completely separates lipid P from inorganic P. Samples of blood or tissue taken a few minutes after injection of inorganic P^{32} show no radioactivity in the lipid fraction when the activity of the inorganic P is highest. The P^{32} was measured in a suitably diluted aliquot; the P^{31} was determined in duplicate aliquots after preliminary ashing with H_2SO_4 and H_2O_2. A large aliquot was evaporated to dryness by aeration at room temperature in the presence of a trace of hydroquinone for gravimetric determination of total lipid. Since at least 90% of the P in the dried lipids was found to be chloroform-soluble and since inorganic phosphate was not present, the alcohol-ether extracts were considered satisfactory for these studies. It must, however, be stated that, if the dried liver residue was extracted with boiling alcohol in the Bailey-Walker extraction apparatus for a 3 hour period, 20 mg more of chloroform-soluble P per 100 g of wet liver were obtained. This fraction, which probably includes the phospholipid attached to nucleoprotein described by Green-

[7] Fiske, C. H. and Subbarow, Yellapragada, *J. Biol. Chem.*, 1925, **66**, 375.

[8] Wang, J. C., Marvin, J. F., and Stenstrom, K. W., *Rev. Scient. Instruments*, 1942, **13**, 81.

[9] Fairbairn, Donald, *J. Biol. Chem.*, 1945, **157**, 645.

[10] Channon, H. J., Platt, A. P., and Smith, J. A. B., *Biochem. J.*, 1937, **31**, 1736.

TABLE II.
Values Obtained with Continuous Intraperitoneal Injection of P^{32} at Rate of 3 Microcuries per Hour.*

1	Hr after starting administration, t		1	2	3	4	6
2	Rats		1	3	2	3	2
3	Body wt, g		202	200	209	199	202
4	Liver wt, % of body wt		3.1	3.7	3.4	3.7	3.5
5	Mg P per 100	Plasma inorganic P	6.4	7.3	6.4	7.1	6.4
6	g liver	Hepatic " "	29.6	26.7	32.9	26.5	31.1
7		" lipid "	144	121	122	129	127
8	Specific activity	Plasma inorganic "	1,127	1,302	1,308	1,673	1,710
9	at time t	Hepatic " "	534	763	1,170	1,509	1,758
10		" lipid "	13	33	102	123	273
11	Relative activity, hepatic lipid: inorganic P		0.024	0.043	0.087	0.082	0.155
12	Mean specific	Hepatic inorganic P	262	470	657	824	1,093
13	activity to	" lipid "	6	15	31	53	105
14	time t	Diff.	256	455	626	771	988
15	Change of specific activity, lipid P per hour		13	17	34	31	46
16		R	0.051	0.037	0.054	0.040	0.046

* The explanation given in the footnote to Table I applies also to Table II.

stein[11] and which requires boiling alcohol for extraction, generally had a little less radioactivity than did the phospholipids extractable with alcohol-ether at room temperature.

Results. Two series of experiments were performed. In the first, which employed the larger number of animals, the radioactive material was injected intravenously in a single dose; in the second the material was administered by continuous intraperitoneal injection. The ratio of liver weight to body weight, the water content, total lipid extractable with alcohol-ether at room temperature, and the concentration of inorganic phosphate of the plasma were similar and within normal limits in all the animals. The chief figures of interest are summarized in Table I for the group of animals that received a single injection and in Table II for the group that received a continuous injection. The corresponding activity-time curves for hepatic inorganic phosphorus and hepatic phospholipid phosphorus were obtained by graphic smoothing of the observed specific activities in order to depict the course of the changes in these values and also for the calculation of the mean activities. The curves are shown in Fig. 1 and 2. The mean specific activity, for inorganic phosphorus and phospholipid phosphorus, which is required for the calculation of R, was obtained by evaluating the area under the time curve and relating this to the time interval considered. The area may be calculated by counting squares on the graph paper, by planimeter, or any other appropriate scheme. We have found most convenient and accurate the division of the area into strips and the use of Simpson's rule. The figures pertinent to the calculation of R are to be found in rows 12 through 16 of the tables. The rate of change of specific activity of hepatic phospholipid per hour (row 15) is obtained by dividing the specific activity at time t (row 10) by the time (row 1). The mean specific activities of hepatic inorganic P and hepatic phospholipid P (rows 12 and 13) were obtained from the area under the time activity curves, as explained previously, and their difference is given in row 14. The value of R (row 16), the proportional turnover rate for the period up to t is

$$R = \frac{\text{Change in specific activity of phospholipid P per hour}}{\text{Mean specific activity of inorganic P} - \text{mean specific activity of phospholipid P}}$$

These were calculated for the first experiment for ½, 1, 2 and 4 hours; the corresponding values were 0.049, 0.052, 0.053 and 0.049 respectively, the last being the rate calculated for the experiment as a whole. The rates calculated for the separate intervals 0 to ½,

[11] Greenstein, J. P., Nucleoproteins, in Anson, M. L., and Edsall, J. T., *Advances in Protein Chemistry*, New York, Academic Press, Inc., 1944, **1**, p. 245.

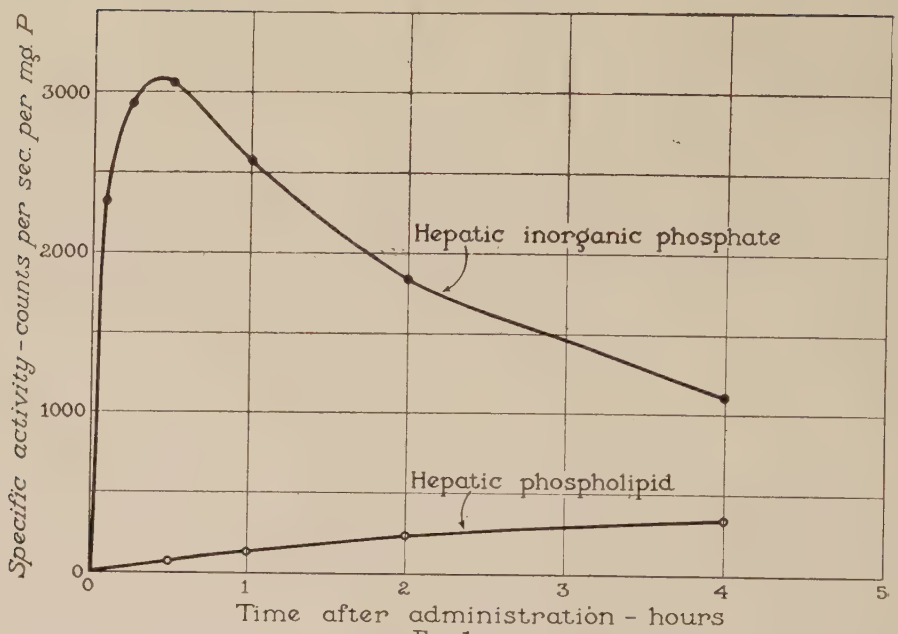

FIG. 1.

Curve of mean values of specific activities found after a single intravenous injection of phosphate P32.

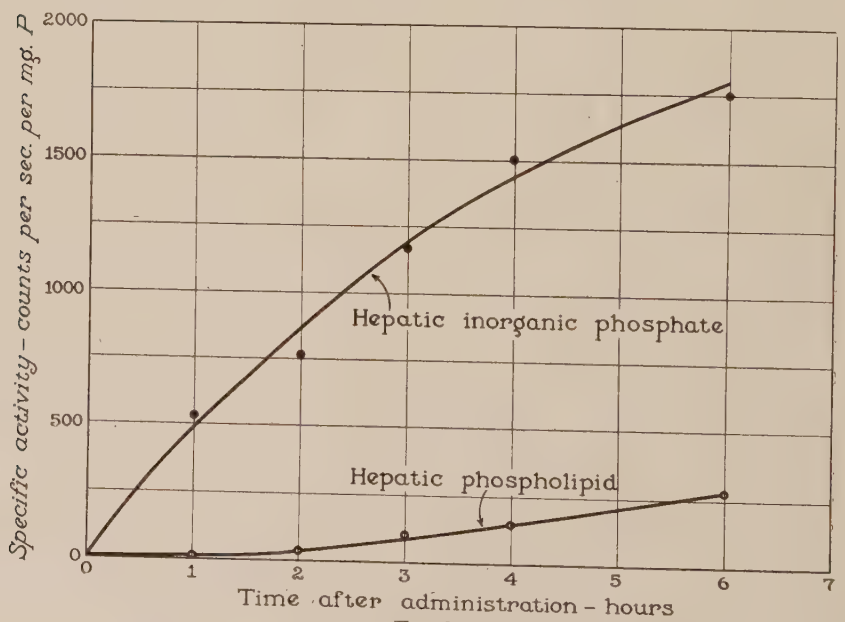

FIG. 2.

Curve of mean values of specific activities found with a continuous intraperitoneal injection of P32.

½ to 1, 1 to 2, 2 to 4 hours were 0.049, 0.052, 0.053, 0.045. It is seen that this experiment yielded very consistent estimates for R in each of the periods. This is in contrast to the relative activity (ratio of specific activity of phospholipid P to that of inorganic P) which rose steadily in this period.

The results for the second series of experi-

ments, in which the injection of the radioactive material was continuous, are shown in Table II and the time-activity curves are shown in Fig. 2. It is to be noted that the curves are markedly different from those of the series with the single injection. This result is to be expected. The calculated values of R are given as for the periods up to 1, 2, 3, 4 and 6 hours; these were respectively 0.051, 0.037, 0.054, 0.040 and 0.046, the last representing the result for the experiment as a whole. The figures are not quite so consistent as those for the first series but the variation is not larger than is to be expected, considering the smaller number of animals used in this series. The relative activity shows no consistency with the first experiment. The figure for R provided by the entire experiment, 0.046, agrees very well with that obtained in the first experiment, 0.049.

Conclusions. The proportional turnover rate R for hepatic phospholipid P in the white rat is on the average close to 5% per hour, and since the mean concentration of hepatic phospholipid P for both experiments together was 132 mg per 100 g of liver, that r, the mass turnover rate, is about 6 mg P per hour per 100 g of liver, which is 0.2 mg P per 100 g body weight.

16287

Growth of Normal Male and Female Mice and of Female Mice Bearing Ovarian Grafts.*†

R. T. HILL. (Introduced by H. G. Day.)

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It has been clearly shown that mouse ovaries, under certain conditions, can supply androgens of sufficient quantity and quality to maintain, in normal conditions, the accessory sex glands of castrate male mice.[1,2] It has been proven that environmental temperature is a factor in causing grafted ovaries to elaborate androgens, but the exact androgens secreted have never been determined.

It was suggested to the author by Doctor Carl G. Hartman that perhaps female mice bearing active androgenic ovarian grafts might assume a growth pattern comparable to that of normal males. Following this suggestion a group of our animals in experiment were weighed twice weekly, and the weights obtained are presented in Fig. 1. Five normal males and 10 normal females were used as control animals. Twenty-eight females bearing autografts of ovaries in their ears comprised the experimental group. All of the animals were of the same age when started on the experiment. It will be noted that the normal males averaged 16.6 g at the outset of the experiment, while the normal and experimental females averaged 14.9 g. The weights were recorded and plotted for approximately 250 days. At the end of this period the plateau of growth was essentially complete.

A difference of slightly less than 2 g at the outset of the experiment, between males and females, had increased to approximately 4.5 g 60 days later. The difference of 4-4.5 g in the weights of the 2 groups was maintained from then till the termination of the experiment. At no time did the curve obtained

* This work has been supported in part by grants from the VioBin Corporation of Monticello, Ill.; the Graduate School and the Medical Center of Indiana University; and the U. S. Public Health Service.

† The mice used in these experiments were the CHI inbred strain obtained from Dr. L. C. Strong.

1 Hill, R. T., *Endocrinology*, 1937, **21**, 633.

2 Hill, R. T., and Strong, M. T., *Endocrinology*, 1938, **22**, 663.

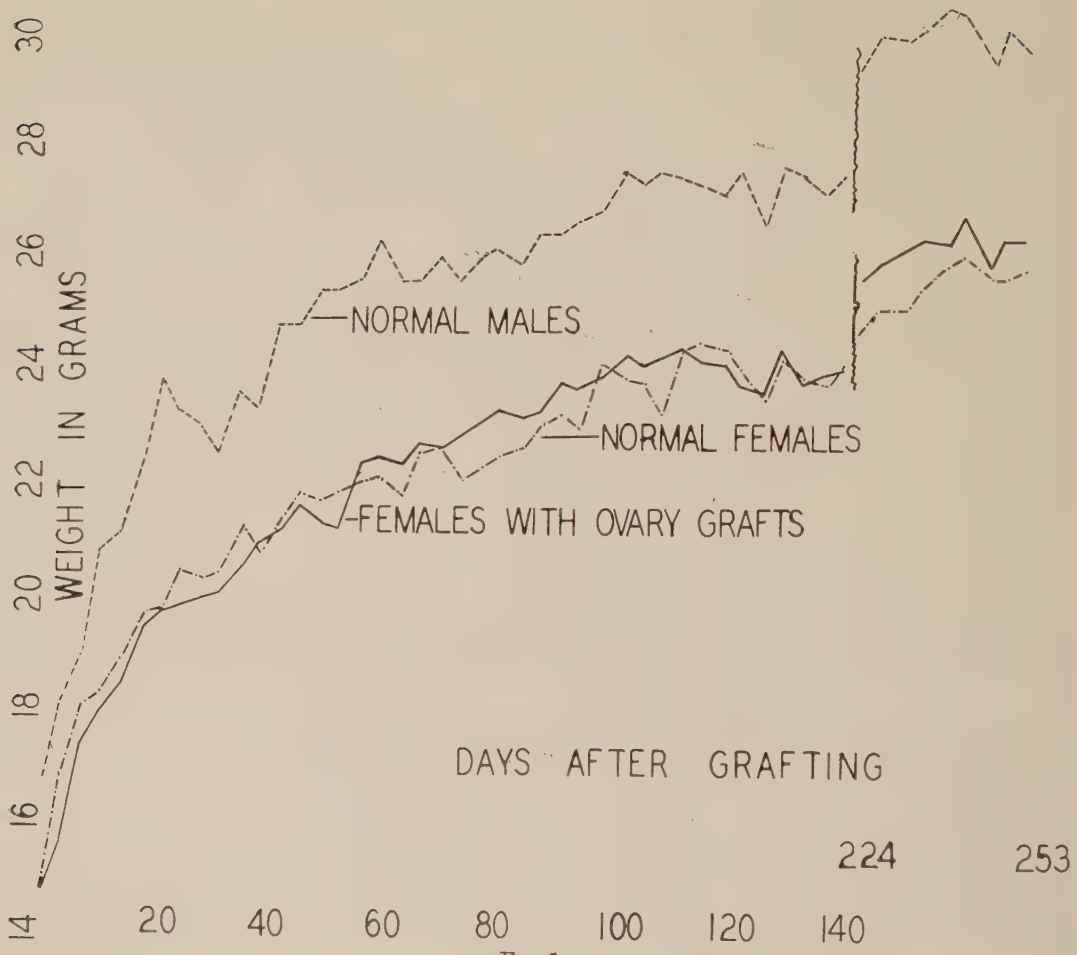

FIG. 1.

Curves representing growth of normal male and female mice and of female mice bearing ovarian grafts.

from the weights of the normal females and that obtained from the grafted females show any significant difference. Likewise, there was no time during the experiment when the growth of the females bearing ovarian grafts even approached the growth of the normal males.

Summary. Grafts of ovaries in female mice which are androgenically active do not affect the growth weights of the host animal.

16288

Storage of Carmine in Mice of Inbred Strains.

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Numerous observations have been reported which suggest a relationship between reticulo-endothelial tissues and the development and growth of malignant tumors (lit. cf.).[1] However, discrepant findings and controversial interpretations have as yet not permitted the formulation of a definite concept.

In the present study the attempt was made to approach this problem by making use of the great variation in spontaneous tumor development exhibited by certain inbred mouse strains. For this purpose, animals of the C3H strain with high mammary cancer incidence and animals of the low-cancer strain C57B were employed. The reticulo-endothelial activity of these animals was assayed on the basis of their ability to store lithium carmine.

Carmine (Merck & Co.) was dissolved in a saturated aqueous solution of lithium carbonate to the desired concentration by boiling gently for 3 minutes, followed by filtration through glass wool. Two, 3 and 4% solutions were employed. They were prepared within the 24 hours prior to use and sterilized in the autoclave for 20 minutes (pressure 10-15 lb). The carmine solutions were injected intraperitoneally with concentration and dosage varying in the 5 experimental groups, as shown in Table I. In the first 3 experiments the animals were sacrificed 48 hours after injection, in the last 2 after 24 hours. Sections of liver and spleen were fixed in 10% formalin; in some groups also skin, kidneys and lungs were preserved. Sections of 6 to 8 micron thickness were cut from the formalin-fixed and paraffin-blocked organs. Of each block, a hematoxylin-eosin stained section and a cleared unstained section were prepared. For general observations, the stained sections were studied. For detailed study of the carmine storage the unstained sections were found best suited.

Particular attention was given to the dye granules taken up by the Kupffer cells in the liver. The over-all impression was that of a more marked storage in Kupffer cells of C57B animals as compared with the findings in C3H mice. In order to express these relations in a roughly quantitative manner, an estimation of the amount of storage was carried out by counting the storing Kupffer cells (SKC) in at least 10 high-power fields and by determining the average of dye-laden cells in each animal. As seen in Table I, this crude quantitative estimation confirmed the impression obtained from general study of the slides.

In all of the 5 experiments, the average numbers of SKC observed in C57B mice exceeded those found in C3H mice. In view of the rather small number of animals making up the single experiments, it is, however, also necessary to analyze the values of SKC in the individual animals. Such an analysis shows that in Experiment I 4 out of 5 C57B mice presented markedly higher SKC values than the 4 C3H mice examined under the same conditions. In Experiment II, 4 out of 6 C57B animals surpassed 6 C3H mice in storing ability. In Experiment III, 1 of the 2 C57B mice showed considerable increase, the other slight increase of SKC as compared with 6 C3H mice. In Experiment IV, the SKC values of 5 out of 6 C57B animals were greater than those obtained in 6 simultaneously tested C3H animals (2 of the latter died 5 hours after the injection of the dye, and hence the small extent of storage probably was due to insufficient time elapsed). In Experiment V, 6 of 7 C57B mice presented higher SKC values than were found in 11 C3H animals under the same conditions. In

[1] Stern, K., and Willheim, R., *The Biochemistry of Malignant Tumors,* Brooklyn, Reference Press, 1943, pp. 696-745.

TABLE I.

			C3H			C57B		
				SKC*			SKC*	
Exp. No.	Carmine conc. and dosage	Time interval in hrs	No. of animals	Values	Mean	No. of animals	Values	Mean
I	3% 0.5 cc (15 mg)	48	4	31		5	54	
				38			67	
				41	38		67	69
				43			76	
							82	
II	2% 0.25 cc (5 mg)	48	6	16		6	22	
				22			35	
				22	25		50	50
				23			60	
				32			62	
				32			71	
III	2% 0.5 cc (10 mg)	48	6	13		2	27†	
				15			41	
				20	19			34
				20				
				21				
				22				
IV	2% 0.5 cc (10 mg)	24	6	5‡		6	24	
				5‡			53	
				10	13		60	69
				10			73	
				19			92	
				26			109	
V	4% 0.5 cc (20 mg)	24	11	10		7	31	
				15			51	
				17			57	
				22			62	
				28			63	
				33	29		64	56
				37			67	
				37				
				38				
				39				
				39				

* Storing Kupffer cells.
† Animal died after 30 hr.
‡ Animal died after 5 hr.

all, 20 out of the 26 examined C57B mice surpassed in carmine storing activity the 33 simultaneously tested C3H mice.

In addition to these quantitative findings, a qualitative difference was frequently noted: finely granular and pale red carmine deposits prevailed in 28 of the 33 C3H mice, with only 5 of them showing dark red, coarsely granular storage, while among the 26 C57B mice 14 presented dark red, coarse granules and 12 exhibited pale red, fine granules. In general, particle size and color intensity tended to increase with the concentration of dye used, though Table I shows that no exact parallelism existed between dye dosage and SKC values. In livers of C3H mice frequently diffuse distribution of dye in sinusoids was noted. Staining of parenchymatous liver cells (diffuse cytoplasmic stain and darker nuclear stain) occurred in both strains only in isolated areas of necrosis encountered infrequently. The intravital carmine staining of liver cells reported by Kiyono[2] was accomplished only by repeated dye injections.

No influence of age or sex was apparent in

2 Kiyono, K., *Die vitale Karminspeicherung*, Jena, Gustav Fischer, 1914.

relation to the findings just described. The age of the animals ranged from 7 weeks to 10 months; for each experimental group animals of similar age were selected from both strains. The sex distribution was as follows: 17 males and 9 females among the C57B animals; 14 males and 19 females among the C3H animals.

In addition to the liver, the carmine storage was studied in the spleens of all animals. Here, too, a different behavior of the two strains, though less constant and striking, was noted: among C57B animals storage was absent in 2, slight to moderate in 17, and marked in 7; among C3H animals storage was absent in 12, slight to moderate in 21, and marked in none. The storage in the spleen was found mainly in the perifollicular reticulum cells and, to a lesser extent, in littoral cells of the sinuses. Thus halo-like formations around the follicles, consisting of dye-laden cells, resulted from the storage, a distribution which did not lend itself readily to quantitative evaluation. The dye storage in the renal epithelium and the dye excretion within the tubules conformed to the classic descriptions[2] and no difference between the two strains was detected. Likewise no appreciable difference was observed regarding dye deposits in skin and lungs, which were examined in some groups of animals.

The fact that in these experiments the liver, *i.e.*, the storage in Kupffer cells, presented the most marked difference between the two strains may be due to the route of administration employed, namely, the intraperitoneal injection of carmine. In this procedure, most of the dye is probably absorbed by serosal lymphatics and thus, by way of the portal vein, reaches the liver first. Intravenous injection, which was not used in this study, may possibly lead to different findings.

In previous work at the State Institute for the Study of Malignant Diseases, Buffalo, N.Y.,* similar experiments were carried out by comparing the carmine storage in C57B mice and in animals of the Marsh-Albino strain. Six animals of each strain were examined. They presented a marked difference in their dye storage in Kupffer cells and in the spleen: heavy, coarsely granular storage was obtained in 5 of the 6 C57B mice and only in 1 of the 6 MA mice.

The C3H and the MA strains are both known for the high incidence of spontaneous mammary carcinoma in the females. On the other hand, this type of tumor is exceedingly rare in the C57B strain. In the latter, the total incidence of all epithelial and non-epithelial spontaneous tumors is around 20% according to studies and records of the Jackson Memorial Laboratory.[3] At this time it would be premature to speculate on a possible relationship between susceptibility or resistance to spontaneous cancer development, on the one hand, and the reticulo-endothelial activity of the strain, as expressed in dye storage, on the other. In order to establish such a relationship it will be necessary to extend this work to a number of additional inbred strains with varying rates of spontaneous tumor development.

Summary. The storage of intraperitoneally injected carmine in Kupffer cells of the liver and in the spleens was examined in mice belonging to two inbred strains. Quantitative and qualitative differences were observed which indicated an inferior dye-storing ability of C3H animals as compared with C57B mice.

* I wish here to thank Dr. A. A. Thibaudeau and Mr. E. Burke, Department of Pathology, and Dr. S. G. Warner, Mr. M. A. Reinhard, and Miss H. Goltz for their cooperation in providing material and facilities for the work done in Buffalo.

[3] Staff of the Jackson Memorial Laboratory, *Biology of the Laboratory Mouse,* The Blakiston Company, Philadelphia, 1941, p. 266.

16289

Mitotic Activity in Male and Female Rat Hypophyses After Combined Injections of Androgen and Estrogen.*

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Since mitotic activity in the hypophysis of female rats is increased significantly after injections of the female hormone estrogen,[1] the question arises whether the male hormone might not likewise affect the proliferative activity in the hypophysis of either male or female animals. There is some evidence that androgen inhibits the activity instead of stimulating it. Wolfe and Hamilton[2] found that the pituitaries of rats were only slightly increased in weight after 10 daily injections of both testosterone-acetate and estrone whereas the increase was considerable after the same number of injections of estrone alone.

In the present experiments combined injections of androgen and estrogen were likewise made into rats to see if there is a modification in mitotic activity of the anterior hypophysis. Also the mitotic activity of male rats is compared with that of female rats after equivalent injections of estrogen.

In determining the mitotic activity, all dividing cells are counted in one or more 3 micra coronal sections of the hypophysis and the number present per square mm of section is calculated. The androgen and estrogen used is testosterone propionate and alpha-estradiol benzoate, respectively, both of which were dissolved in sesame oil.‡ All injections were made subcutaneously. The female animals were ovariectomized approximately one month previous to the time of injections.

The tabulation of the experiments shows the number of animals, the average age of the animals in each group, the hormones injected and the mean and standard error of the mean of the mitoses per sq mm of section. Injections of estrogen were made either 48 and 72 hours before killing the animals or, as in Groups 5, 6, and 7, they were made 28, 36, 52, and 60 hours before killing. In Groups 2 and 3 the androgen was injected 24, 48, and 72 hours before killing, and in Group 6 it was injected at the same times as the estrogen.

The number of dividing cells in the hypophysis of ovariectomized females and in that of normal males is uniformly low in animals 3 months of age or older (Groups 1 and 8). Only 2 or 3 mitotic figures are usually seen and at the most 5 to 10 occur in an entire section that includes 5 or 6 square mm.

Injections of estrogen result in a considerably increased mitotic activity in both ovariectomized females and normal males (Groups 4, 5, and 7). In all 3 groups the average number of mitoses is about 20 per sq mm. Statistically there is no significant difference in the mitotic activity of the male Group 7 and female Group 5, both of which received estrogen ($P = .8$).§ In the males there is no evidence that the testicular androgen modifies the activity of the injected estrogen. The male hypophysis thus appears to respond to the estrogen about the same as that of the female. However, in the male there is a higher percentage of chromophil cells undergoing division than there is in the female, although, as in the female, the majority of divisions occur in the chromophobes.

Injections of androgen into ovariectomized

* This study was aided by a grant from the Cancer Research Division of the Donner Foundation.

† With the technical assistance of Miss Cecile Morgan.

1 Hunt, T. E., *Anat. Rec.*, 1947, **97**, 127.

2 Wolfe, J. M., and Hamilton, J. B., *Endocrinology*, 1937, **21**, 603.

‡ The estrogen (Progynon-B) and androgen (Oreton) were kindly furnished by the Schering Corporation, Bloomfield, N.J.

§ Fischer's table of P was used to determine statistical significance. P must have a value of less than .05 before a difference can be considered as possibly significant.

TABLE I.
Mitotic Activity in the Hypophysis of Rats.

Group	No. of animals	Age mean	Sex	Inj. of androgen and/or estrogen μg	Mitoses per mm² Mean ± S.E.
1	5	117	♀		1.20 ± 0.25
2	4	102	♀	3x500A	1.17 ± 1.07
3	6	101	♀	3x500A + 2x25E 3x1000A + 2x25E	17.0 ± 2.76
4	10	97	♀	2x25E	20.0 ± 1.94
5	10	91	♀	4x17E	21.3 ± 1.55
6	6	94	♂	4x500A + 4x17E	18.7 ± 4.20
7	6	93	♂	4x17E	20.8 ± 2.00
8	9	92	♂		.52 ± .13

females does not change the mitotic activity in the hypophysis (Group 2). The glands of only 4 animals were examined but since in all of these the activity is uniformly low, it seemed unnecessary to extend the series.

In the males and females which received both androgen and estrogen (Groups 3 and 6) the average mitotic activity does not differ significantly from either the male or female groups which received estrogen alone. In comparing Group 3 with 4 statistically, P = .35, and 6 with 7, P = .65. The animals in Group 3 that had injections of 500 μg of androgen show a somewhat lower activity than those which had 1000 μg, but the difference is not believed to be important.

Summary. The estrogenic hormone, alpha-estradiol benzoate, stimulates mitotic division in the cells of the anterior hypophysis of both male and female rats and, provided the animals are about the same age, an equal amount injected results in approximately the same mitotic activity. When the androgen, testosterone propionate, is injected with the estrogen in either male or female rats, the mitotic activity is not significantly different from that resulting from injections of estrogen alone.

16290 P

Increased Susceptibility of Mice to Swine Influenza as a Result of Methionine Injections.*

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In recent years numerous experiments have been reported on the effects of various dietary constituents on the susceptibility of animals to viral infections. I reported in 1942 that methionine decreased the susceptibility of the rabbit to vaccinia.[1] It seemed advisable however to repeat these experiments in the mouse using swine influenza as the virus. When mice on an adequate diet are injected with methionine there is a slight increase rather than decrease in susceptibility to swine influenza. But, if methionine were given to mice on a low protein diet, a marked increase in susceptibility occurs. This paper is to report such experiments.

Since poor or malnutrition from many causes will result in an increase in the animal's resistance to viral infections[2] it was thought

* Aided in part by grants-in-aid from The John and Mary R. Markle Foundation and the United States Public Health Service.

1 Sprunt, D. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 226.

2 Sprunt, D. H., *J. Exp. Med.*, 1942, **75**, 297; Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **80**, 257.

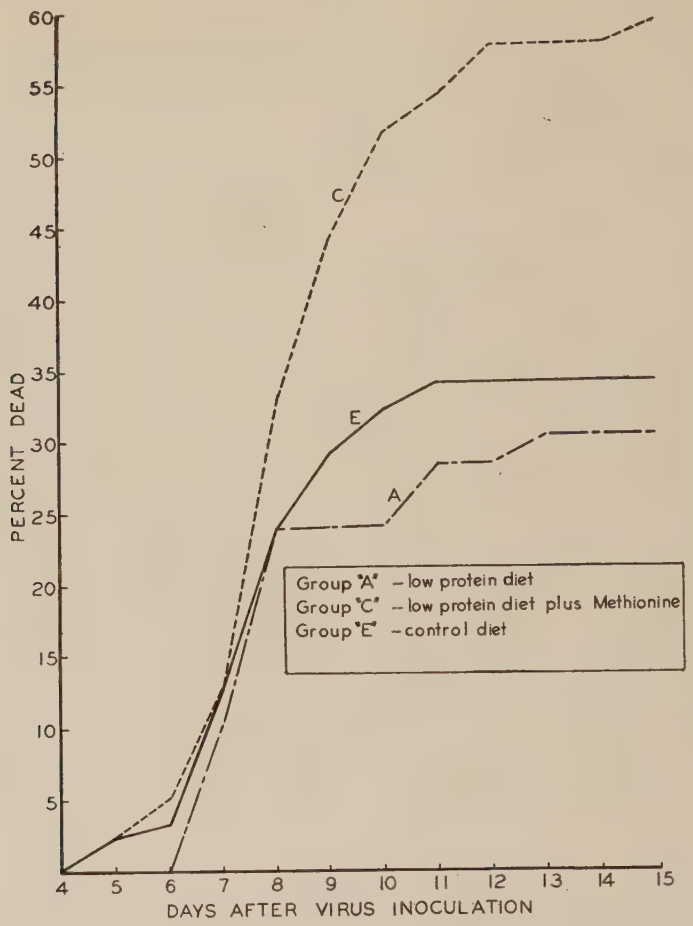

CHART 1.
The percentage of mice on different diets dying after injection with swine influenza.

essential that the substance to be studied should be added to a diet which by itself caused some malnutrition and increased resistance. A low protein diet was found to meet these requirements. Although malnutrition generally increases the animal's resistance to infection it does, if too extreme, result in the death of the animals from starvation. For this reason mice under similar conditions to those injected were kept as controls, in order to see if any of the deaths were due to malnutrition.

In the following experiments 360 male adult mice weighing approximately 25 g each were divided into 6 groups. The mice in Groups A, B, C, and D were fed the low protein diet, but in addition Groups C and D were given daily intraperitoneal injections of 15 mg of an aqueous solution of methionine. After 2 weeks the mice in Groups A and C were inoculated intranasally, under ether anesthesia, with swine influenza virus.† The virus was diluted before inoculation to the 50% point, although similar results could be obtained with other strains of both human and swine influenza virus. This virus was used in most of the experiments, since the 50% point was more constant in various groups of mice. In these experiments it is

† The virus was kindly supplied to us by Dr. Joseph Beard of Duke University. It was an egg-adapted strain of swine influenza which came originally from Dr. R. E. Shope and has been designated as Strain 15.

TABLE I.
Amount of Weight Lost by Mice on the Various Diets.

Groups	A	B	C	D	E	F
	Avg wt in g					
At start of experiment	24.4	24.6	24.0	23.9	23.0	25.4
At time inoculated with virus	21.7	22.1	21.7	21.4	24.2	25.2
At time of death	15.9	—	16.6	—	16.8	—
Surviving mice	20.3	22.6	19.1	21.0	25.1	26.7

essential that the virus dilution be kept at this 50% point in order to assure a number of survivors. Groups B and D remained under similar condition but received no virus. The 4 groups did not differ in food intake and weight. Rockland Mouse Diet which is adequate and has a protein level of 24% was fed the mice in Groups E and F. Inoculation of the mice in Group E was done at the same time and in the same manner as in Groups A and C. The controls, the mice in Group F, received no virus.

The results of the experiment are given in Chart 1. Groups B. D, and F are omitted as none of the mice died during the experiments. The weights of all the groups are shown in Table I. It is seen that all the animals in Groups A, B, C, and D lost similar amounts.

The mice on the low protein diet plus methionine are the most susceptible to swine influenza, those on the low protein diet alone the least susceptible. The increased susceptibility of the mice receiving methionine over the other 2 groups is significant statistically. The differences between the mice on the Rockland Mouse Diet and on the low protein diet has been significant in some experiments but not in others.

Six experiments similar to the above and others along the same lines have given similar results. Cystine used under similar conditions increased slightly the susceptibility of the mouse to swine influenza. The effect of other amino acids is now being studied.

We have no explanation as to why methionine decreased the susceptibility of the rabbit to vaccinia and increased the susceptibility of the mouse to swine influenza. The experiments which we have in progress may elucidate this problem. Three possibilities appear now: (1) Methionine in some way lowers the resistance of the host to infection, possibly by increasing the permeability of the cell to the virus. (2) Methionine may interfere with the formation of antibodies. (3) Multiplication of the virus may be aided by methionine.

Conclusions. Mice on a low protein diet, if given methionine, have a greatly increased susceptibility to the swine influenza virus.

16291 P

Effect of Adrenalectomy Upon Level of Blood Amino Acids in the Eviscerated Rat.

DWIGHT J. INGLE, MILDRED C. PRESTRUD, AND JAMES E. NEZAMIS.
From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Following evisceration the concentration of amino acids in the blood increases gradually throughout the period of survival but this rise can be completely suppressed by the administration of optimal amounts of insulin with glucose.[1] In the present study it was shown that adrenalectomy suppressed the level of blood amino acids below that of non-adrenalectomized eviscerated rats at each

[1] Ingle, D. J., Prestrud, M. C., and Nezamis, J. E., *Am. J. Physiol.*, 1947, **150**, 682.

level of insulin dose and glucose load.

Methods. Male rats of the Sprague-Dawley strain were eviscerated at 250 g weight. Adrenalectomy was performed in the same operation. Immediately following operation all of the animals were given continuous intravenous infusions of 0.9% sodium chloride solution with and without added glucose and crystalline zinc insulin (Lilly). The volume was 20 cc per 24 hours per rat. The details of the methods have been described.[1]

Experiments and Results. Equal numbers of eviscerated rats and adrenalectomized-eviscerated rats were studied simultaneously. Samples of blood were taken from the abdominal aorta at the end of 24 hours. Sixteen pairs of rats were given 4 units of insulin and 40 mg of glucose per 100 g of rat per hour (40/100/h); 10 pairs were given 1 unit of insulin and 40/100/h; 16 pairs were given 0.5 units of insulin and 24/100/h; and 7 pairs were given no insulin and 8/100/h of glucose. At each level of insulin dosage and glucose load the average value for amino acids was lower in the adrenalectomized-eviscerated rats than in the eviscerated rats. Likewise, in each of the 49 pairs of rats the value for blood amino acids was lower in the adrenalectomized rat than in the non-adrenalectomized rat. The data are summarized in Fig. 1.

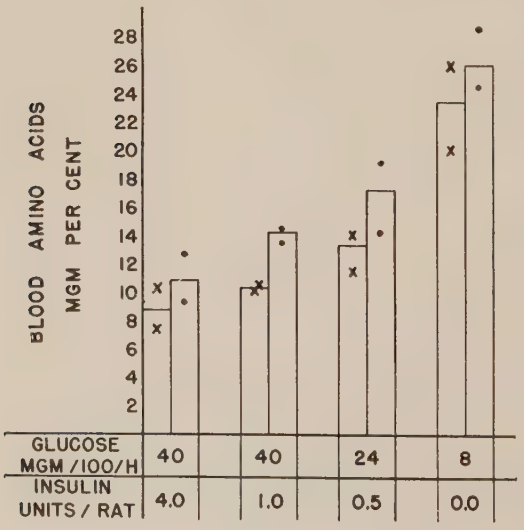

FIG. 1.

Level of blood amino acids at the end of 24 hours of continuous intravenous infusion. Averages and Range.

• Eviscerated rats.

x Adrenalectomized-eviscerated rats.

Discussion. These results are consistent with an earlier study showing that insulin prevents the rise in blood amino acids which follows evisceration in the rat. The effect of adrenalectomy upon the level of blood amino acids in the eviscerated rat is apparently independent of insulin dosage and glucose load. Friedberg and Greenberg[2] have shown that the level of plasma amino acids is depressed by adrenalectomy in the non-eviscerated rat. Roberts[3] reported that adrenalectomy in the eviscerated rat did not influence the rate of rise of blood amino acids but the periods of observation were shorter than in the present study and other conditions were different.

How do these data relate to the subnormal ability of the untreated adrenalectomized animal to mobilize its cellular proteins during stress? The level of blood amino acids in the eviscerated animal represents a balance between the breakdown and resynthesis of tissue proteins the rates of which were not appraised in this study. What would be the effect of cortical hormone overdosage upon the level of blood amino acids in eviscerated animals? Cortical hormone overdosage is antagonistic to the hypoglycemic effect of insulin in the normal force-fed rat[4] and the eviscerated rat.[5] Would it antagonize the effect of insulin in suppressing the rise in blood amino acids which follows evisceration? Further studies are planned.

Summary. Adrenalectomy in the eviscerated rat lowered the level of amino acids in whole blood at each level of insulin dosage and glucose load when determined 24 hours after operation.

2 Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, 1947, **168**, 405.

3 Roberts, S., *Endocrinology*, 1946, **39**, 80.

4 Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinology*, 1945, **37**, 341.

5 Ingle, D. J., Prestrud, M. C., Nezamis, J. E., and Kuizenga, M. H., *Am. J. Physiol.*, 1947, **150**, 423.

16292

Effects of B Vitamins, Yeast and Liver on Ovaries of Immature Rats Fed Massive Doses of Alpha-Estradiol.

B. H. Ershoff and H. B. McWilliams.

From the Emory W. Thurston Laboratories, Los Angeles, California.

It is well established that massive doses of estrogens retard growth and inhibit gonadal development in the immature rat. Available data indicate that these effects may be counteracted, at least in part, by dietary means. Ershoff and Deuel[1] observed that the gonadal weight of immature rats fed massive doses of alpha-estradiol was significantly greater on diets containing yeast than on rations containing the B vitamins in synthetic form. In the present communication further data are presented on the effects of diet on ovarian development in the alpha-estradiol-fed rat.

Procedure and Results. Four basal rations were employed in the present experiment: diets A, B, C, and D. Diets A and B were purified rations containing the B-complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. All 4 rations were supplemented with 0.0 and 10.0 mg of alpha-estradiol per kg of diet.* Sixty-four female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 42.0 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed *ad lib.* the diets listed in Table I. Feeding was continued for 8 weeks. Animals were autopsied on the 56th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

Results are summarized in Table II. In agreement with earlier findings[1] the effects of alpha-estradiol feeding in the immature rat were dependent on the diet employed. On the synthetic ration (diet A_2) ovaries appeared infantile both in weight and microscopic appearance. Similar results were obtained in alpha-estradiol-fed rats receiving additional B vitamins or yeast (diets B_2 and C_2). In the whole liver series (diet D_2), however, ovarian weights averaged approximately twice the values obtained on other alpha-estradiol-containing rations; and histologically ovaries appeared normal in 8 of the 10 rats in this series. Growth was markedly reduced in all rats fed alpha-estradiol-containing rations. Gain in body weight was somewhat greater in the liver series (diet D_2) than on other rations employed, but with the possible exception of the A_2 series these differences were not significant. On alpha-estradiol-free rations body and ovarian weights did not differ significantly on any of the diets employed, and histologically ovaries appeared normal in all groups.

Discussion. It is becoming increasingly apparent that the effects obtained in an experimental animal following administration of drugs or related products are dependent on the nutritional state and the diets employed. In acute deficiencies of essential nutrients it is readily recognized that resulting abnormalities in cellular metabolism may profoundly affect response to certain drugs. What is less well recognized, however, is that these drugs themselves may precipitate a deficiency state. Thyroid administration, for example, may induce a deficiency of thiamine, pyridoxine, and pantothenic acid,[3] while dicumarol, salicylates, and other drugs may precipi-

[1] Ershoff, B. H., and Deuel, H. J., Jr., *Am. J. Physiol.*, 1946, **145**, 465.

* The alpha-estradiol for these experiments was obtained from the Schering Corporation, Bloomfield, N.J. One mg alpha-estradiol ≃ 12,000 R.U. or 120,000 I.U. estrone.

[2] Sure, B., *J. Nutrition*, 1941, **22**, 499.

[3] Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1942, **135**, 474.

[4] Shapiro, S., Redish, M. H., and Campbell, H. A., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 12.

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A_1 and A_2	Diet B_1 and B_2	Diet C_1 and C_2	Diet D_1 and D_2
Yeast*	0.0	0.0	10.0	0.0
Whole liver powder†	0.0	0.0	0.0	10.0
Casein‡	22.0	22.0	22.0	22.0
Salt mixture§	4.5	4.5	4.5	4.5
Sucrose	73.5	73.5	63.5	63.5

Alpha-estradiol was incorporated in diets A_2, B_2, C_2, and D_2 at a level of 10.0 mg per kg of diet.

To each kg of diet A, C, and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg, and choline chloride 1.2 g.

To each kg of diet B were added: thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, inositol 1.2 g, *p*-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-naphthoquinone 10 mg and choline chloride 1.2 g.

Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A-D concentrate‖ containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

* Brewer's Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

† Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

‡ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Sure's Salt Mixture No. 1.[2]

‖ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

TABLE II.
Effects of Alpha-estradiol on Body and Ovarian Weight in the Immature Rat.

Dietary Group	No. of animals	Initial body wt, g	Gain in body wt 8-wk period,* g	Avg ovarian wt,* mg
		Alpha-estradiol Series.		
A_2	10	42.8	97.9 ± 3.7	13.7 ± 1.1
B_2	10	42.1	105.1 ± 6.6	16.1 ± 1.3
C_2	10	41.9	103.4 ± 5.5	18.9 ± 1.4
D_2	10	42.2	117.9 ± 3.6	37.2 ± 2.7
		Control Series.		
A_1	6	41.7	146.4 ± 9.0	47.0 ± 2.4
B_1	6	42.0	152.3 ± 7.8	49.1 ± 3.0
C_1	6	41.5	146.8 ± 6.9	43.2 ± 3.2
D_1	6	41.7	169.7 ± 9.3	44.8 ± 2.1

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

tate a deficiency of vitamin K.[4-6] In addition to the known nutrients, however, requirements for various unknown factors may also be increased following the administration of certain drugs. These "minor vitamins" are apparently dispensable under normal conditions or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriatee amounts of the missing nutrient.[7] Whole liver and yeast are potent sources of such unknown nutrients. The beneficial effects of these latter substances in animals inhaling carbon tetrachloride or fed toxic

5 Shapiro, S., *J. A. M. A.*, 1944, **125**, 546.

6 Collins, E. N., and Hoffman, A. D., *Cleveland Clin. Quart.*, 1943, **10**, 105.

7 Ershoff, B. H., *Physiol. Rev.*, 1948, **28**, 107.

doses of strychnine, promin, dinitrophenol, sulfanilamide, atabrine, and other drugs have been recognized for years.[8-11] Similar results have been observed following toxic doses of diethylstilbestrol[8] and desiccated thyroid.[12-14] In the present experiment toxic effects of alpha-estradiol were similarly modified by dietary means.

Findings indicate that the effects of alpha-estradiol feeding in the immature rat were dependent on the diet employed. On the synthetic ration (diet A_2) ovaries appeared infantile both in weight and microscopic appearance. Similar results were obtained with animals fed additional B vitamins or yeast (diets B_2 and C_2). Oral administration of desiccated whole liver, however, resulted in a significant increase in ovarian weight with ovaries resembling histologically those of the normal rat. On alpha-estradiol-free rations ovaries did not differ significantly in weight or microscopic appearance on any of the diets employed. Available data indicate that the beneficial effects of liver on ovarian development in the immature alpha-estradiol-fed rat were not due to any of the known B vitamins. This is indicated by the fact that ovaries remained infantile on diet B_2 although this ration contained all known members of the vitamin B complex in amounts exceeding their presence in the liver-containing diet D_2. It is suggested, therefore, that desiccated whole liver contains some factor(s) other than the known B vitamins whose requirement is increased following prolonged feeding of alpha-estradiol.† In previous work with smaller doses of alpha-estradiol yeast was also found to promote ovarian development in the alpha-estradiol-fed rat.[1] Such was not the case, however, under conditions of the present experiment.

Summary. Desiccated whole liver counteracted the inhibition of ovarian development observed in immature rats fed massive doses of alpha-estradiol. The protective factor in liver was distinct from any of the known members of the vitamin B complex. It was not present in significant amounts in yeast.

8 Chamelin, I. M., and Funk, C., *Arch. Biochem.*, 1943, **2**, 9.

9 Higgins, G. M., *Am. J. Clin. Path.*, 1944, **14**, 278.

10 Battelli, G., *Boll. soc. ital. biol. sper.*, 1940, **15**, 687.

11 Ershoff, B. H., *J. Nutrition*, 1948, **35**, 269.

12 Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

13 Ershoff, B. H., *Arch. Biochem.*, 1947, **15**, 365.

14 Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

† The unknown nutrient may be part of an enzyme system concerned with estrogen inactivation. It is possible that prolonged feeding of alpha-estradiol increased requirements for this factor on diets A_2, B_2, and C_2 to the point that a deficiency occurred, interfering with estrogen-inactivation and resulting in impaired secretion of pituitary gonadotropins.[15]

15 Zondek, B., *Clinical and Experimental Investigations on the Genital Functions and Their Hormonal Regulation*, Williams & Wilkins, Baltimore, 1941.

16293

The Ability of the Cat to Withstand Repeated Electrically Induced Convulsions.

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In carrying out electro-shock therapy on psychotic patients it is frequently observed that some fail to immediately respond to the electrical stimulus with a major convulsive seizure. At times, especially in attempting to establish the convulsive threshold for a particular patient, it becomes necessary to induce 3 or 4 minor responses before the

desired major reaction becomes evident. In other patients, especially in those who are markedly disturbed, it has been observed that a single convulsion may fail to induce adequate sedation. The safety of repeated convulsions in such overly-disturbed patients has, therefore, been considered. Since a survey of the literature has failed to disclose the tolerance of animals to repeated electrically-induced convulsions and because of a fear of the consequences of repeated electrical stimulation this procedure had to be deferred.

Because of a need for such information it was deemed advisable to study this problem experimentally.

For this purpose 11 adult cats (exact ages unknown) were subjected to repeated electrical stimulation at approximately five-minute intervals. In 8 animals (Group A) this procedure was continued until each animal expired. The other 3 cats (Group B) were permitted to recover after having been subjected to a prolonged series of convulsions. On the basis of experience with patients submitted to electroshock this procedure was deemed entirely painless both at the time the convulsions were induced and after the convulsions were over.

The apparatus used to deliver the current was similar to that employed on patients. This utilized the 60-cycle 120-volt house current and consisted of a *step-up and step-down transformer* operated by an electrical timing switch. These were so designed that the duration of the current flow and voltage could be varied by the operator (Lektra Laboratories, New York). The lower voltage range was especially calibrated thus permitting a more accurate determination of the voltage required for the small animal used. The duration of the stimulus was maintained at a constant of 0.2 seconds. The electrodes used were especially constructed to better conform to the small animal head (Lektra Laboratories, New York). In each instance the convulsion was induced by the minimal convulsive dose and this had to be gradually increased in each animal as the experiment progressed.

Results. These have been tabulated in Tables I and II. From Table I it may be

TABLE I.

Tabulation of Data in Electrically-induced Convulsions in the Cat. Stimuli were repeated at 3.5-5.5-minute intervals and the duration of each stimulus was 0.2 seconds.

	Group A (Expired)									Group B (Survived)		
Cat Number	8	15*	16*	19	20*	21*	22*	24*	Avg	17*	18*	23
Wt in lbs	7¾	5¼	4¼	4¼	4¾	6½	5¾	5		8	7	5
Major reactions	30	64	60	46	53	49	75	87	58	40	49	95
Minor reactions	26	20	31	2	9	8	12	7	14.4	22	4	10
Duration of experiment, hr and min.	4-40′	5-50′	6-56′	4-44′	5-23′	5-0′	7-40′	8-19′	6-4′	5-25′	5-17′	9-0′
Initial voltage	80	90	50	40	40	40	40	40	52.5	50	50	40
Terminal voltage	140	150	190	80	90	80	140	150	127.5	120	80	160
Initial amperage (in ma.)	160	100	100	57	57	50	57	44	78.12	71	71	50
Terminal amperage (in ma.)	166	333	475	133	103	177	311	300	278.25	228	160	355
Coulombs ($Q = IT$)		1.83	3.65	5.24	0.91	0.99	1.30	3.20	3.23	2.54	1.85	1.22	4.28
Q/lbs		0 24	0.70	1.22	0.22	0.21	0.20	0.55	0.65	0.50	0.23	0.17	0.85

* Brain material available.

TABLE II.
Comparison of Original Minimal Convulsive Dose (M.C.D.) of Electricity (Q_2) with Total Current Employed (Q_1).

	Group A (Expired)								Group B (Survived)		
Cat Number	8	15	16	19	20	21	22	24	17	18	23
Total coulombs (Q_1)	1.83	3.65	5.24	0.91	0.99	1.30	3.20	3.23	1.85	1.22	4.28
Coulombs of M.C.D. (Q_2)	.032	.020	.020	.010	.010	.010	.010	.008	.032	.014	.010
Q_1/Q_2	57	132	262	91	99	130	320	403	57	87	428

seen that of those animals which expired (Group A), 1 succumbed after 30 convulsions; 1 at the end of 87 convulsions; and that the average number of convulsions was 58.

In addition, a varying number of minor (non-convulsive) reactions was observed—these occurring for the most part between major reactions when the animal failed to respond adequately to the previously effective minimal convulsive dose.

The total duration of treatment in this group varied from 4 hours 40 minutes to 8 hours 19 minutes (average = 6 hours, 4 minutes).

Initial minimal convulsive voltage of 40-90 volts (average = 52.5 volts) was gradually observed to rise to 80-190 volts (average = 127.5 volts) as the experiment progressed.

Initial minimal convulsive amperage of 44-160 ma. (average = 78.12 ma.) was gradually observed to rise to 103-475 ma. (average = 287.25 ma.).

The total current in coulombs (Q) administered to each animal (according to $Q = IT$) was found to vary from 0.91 to 5.24 coulombs (average = 2.54 Q).

In those animals surviving (Group B), it will be observed that one animal had had as many as 95 convulsions; that the total duration of the experiment in this animal was 9 hours; and that the total current administered to this animal was 4.28 coulombs. In spite of such intense treatment the rapidity of recovery, as judged by hopping and placing reactions, gait, feeding habits, preening and general alertness, was truly amazing. After 21 days these cats were behavioristically indistinguishable from untreated controls.

Discussion. It will be observed that in speaking of current intensities these were always designated "currents administered" rather than as "currents received" by the animal. This distinction was made in order to make clear that exact amounts of current received by the animal brain remained unknown since the degree of dissipation along skin and bone was not ascertained.

It must be admitted that the number of convulsions through which these animals survived was surprisingly high especially since

they were experienced in such concentrated form. It is interesting that the oldest appearing cat in the group (No. 8) was first to die. The brain of this animal, like those of all others, showed no evidence of gross pathology. Microscopic studies will be reported later. Electroencephalographic changes were observed, however, as the experiment progressed and these consisted for the most part of a gradual slowing and decrease in voltage. Detailed study of these findings is now under way.

The rise of the convulsive threshold as the experiment progresses is also significant in that it is at first very gradual. Later it increases in accelerated fashion so that in some cases the animal requires 4 times the initial voltage and as much as 7 times the initial amperage before responding with a convulsion. This is probably due to exhaustion, and experiments are now being planned to verify this hypothesis.

The striking fact obtained from this study is that no positive correlation between the amount of electricity applied and the tolerance of the animal could be demonstrated. This lack of correlation can best be appreciated from the tabulated coulomb-body weight ratios (Q/lbs) in Table I. These ratios show a variation of from 0.21 to 1.22 total coulombs per pound body weight in those animals which expired; while the surviving animals showed variations in ratios which were almost as great. This seems to indicate that of those cats which expired some required almost 6 times as many coulombs as others. It, therefore, appears that tolerance of electrical stimulation by a particular animal varies for each animal as an individual. Perhaps the best criterion of the ability of the cat to withstand electrical stimulation is illustrated in Table II. Here, a comparison of the original convulsive quantity of current (Q_2) with the total coulombs used (Q_1) discloses that in those cats expiring this ratio varied from 57 to 403. In the 3 surviving cats these ratios were 57, 87, and 428 respectively. In other words, of all the cats used it required at least 57 times the minimal convulsive coulomb value before any of them died. And in one instance a cat survived 428 times the original minimal convulsive coulomb value.

Summary. Adult cats submitted to repeated stimulations with, whenever possible, minimal convulsive doses of electricity showed an ability to withstand 30 to 87 major convulsions before expiring. Of those cats surviving the experiment one experienced 95 such convulsions. Initial voltage was observed to rise in one instance to approximately 4 times, and initial amperage was observed in one instance to rise to approximately 7 times the original minimal convulsive levels as the experiment progressed. The oldest appearing cat in the group was first to succumb. The brain of this cat, like the brains of all others, showed no *gross* pathological changes. Electroencephalographic changes in the form of gradual slowing and decreasing voltage were observed as the experiment progressed. The surviving cats showed no gross defects in behavior 21 days after the last convulsion.

It is *assumed* that death when it occurred was the result of exhaustion. No correlation between total applied current and tolerance could be ascertained. The striking observation is the unexpectedly high tolerance of the adult cat for repeated minimal convulsive-inducing doses of electricity. This is quantitatively best shown in comparisons between original minimal convulsing coulomb values and total coulombs used. In those cats which expired this total varied from 57 to 403 times the original minimal convulsive quantity. Of the surviving cats one received as much as 428 times the original minimal convulsive coulomb value. These experiments, therefore, point to the relative safety of the minimal convulsive dose of electricity in the cat and suggest a probable greater tolerance in the human than has heretofore been recognized.

16294

A Biological Method for Determination of Curare and Erythroidine-Alkaloids.

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Recent studies[1] have shown that mice pretreated with morphine sulfate are especially sensitive to curare and erythrina-alkaloids. Since these alkaloids in microgram amounts antagonized both excitement and the characteristic Straub-phenomenon[2] on the tails of morphinized mice before any peripheral paralyzing action became evident, it was of interest to investigate whether this procedure could be employed as a sensitive biological method for the determination of low concentrations of these alkaloids.

Two methods which permit the estimation of curare *in vitro* have been developed which make use of isolated mammalian preparations: one method (Bülbring-Chou[3]) employs the phrenic nerve diaphragm preparation from the rat and tests the curare depressant effect of rapid motor nerve stimulation; the other (P. D. Garcia de Jalon[4]) is based on the well known antagonistic action of curare[5] on the acetylcholine contracture in *M. rectus abdominis* of the frog. These *in vitro* methods provide a rapid check on pure curare preparations, but are less useful for the exact measurement of impure preparations and tissue extracts. The rabbit head drop method of McIntyre and Holaday[6] provides an accurate *in vivo* assay for this type of preparation but requires the use of an appreciable amount of substance. Recently Skinner and Young[7] have proposed a mouse assay which is dependent on the peripheral paralyzing action of the alkaloids as determined in the sloping rotating cylinder apparatus of Young and Lewis.[8]

Method. Groups of 5 to 10 white mice, weighing 18-20 g each, are injected subcutaneously with 0.5 mg of morphine sulfate. Restlessness and the typical tail reflex occur generally within 5 to 10 minutes and persist for 2 hours or more. Doses smaller than 0.5 mg of morphine sulfate are not recommended because the characteristic morphine effect is either delayed or of not sufficient duration. Curare and erythrina preparations, dissolved in 0.85% sodium chloride, are injected intraperitoneally in a volume not exceeding 0.5 cc into animals exhibiting the typical morphine reaction. A positive effect appears within 5 to 10 minutes and is characterized by disappearance of the excitement phenomena and relaxation of the tails. This period lasts usually for 10-15-25 minutes and is followed by the gradual reappearance of restlessness and tail phenomenon. At this point, the mice may again be used for testing without danger of alteration of the alkaloid response since intraperitoneally injected curare is rapidly destroyed or excreted.

Table I shows the results obtained following the injection of graded doses of various curare and erythroidine preparations. From these data it can be determined that the median effective dose and its standard error of *d*-tubocurarine chloride is 2.8 $\pm$ 0.2 μg;

1 Pick, E. P., and Richards, G. V., *J. Pharm. and Exp. Therap.*, 1947, **89**, 1.

2 Straub, W., *Deutsche med. Wchnschr.*, 1911, **37**, 1462; Straub, W., and Herrmann, O., *Biochem. Z.*, 1912, **39**, 216.

3 Bülbring, E., *Brit. J. Pharmacol. and Chemotherap.*, 1946, **1**, 38; Chou, T. C., *ibid.*, 1947, **2**, 1.

4 Garcia de Jalon, P. D., *Quar. J. Pharm. and Pharmacol.*, 1947, **20**, 28.

5 Riesser, O., and Neuschloss, S., *Arch. f. exp. Path. u. Pharmacol.*, 1922, **92**, 254.

6 McIntyre, A. R., and Holaday, H. A., quoted by Bennett, A. E., *Am. J. Psychiat.*, 1941, **97**, 1040.

7 Skinner, H. G., and Young, D. M., *J. Pharm. and Exp. Therap.*, 1947, **91**, 144.

8 Young, D. M., and Lewis, A. H., *Science*, 1947, **105**, 368.

TABLE I.

Graded Doses of Various Curare and Erythroidine Preparations Antagonizing the Effect of Morphine Poisoning on Mice.

Drug	Dose per 20 g mouse*	No. of animals	No. of animals responding with disappearance of morphine symptoms	Duration of curare effect in min.
Cryst. *d*-tubocurarine chloride	2.5 μg	10	5	5-10
	3.0 "	15	10	5-13
	5.0 "	10	10	25-50
Strychnos curare (Merck)	20.0 "	5	0	—
	30.0 "	5	5	12
	40.0 "	5	5	24
Cryst. dihydro-β-erythroidine-bromide	40.0 "	5	0	—
	50.0 "	10	10	18
	60.0 "	10	10	10
Intocostrin (Squibb)	15.0 milliunits	5	—	—
	20.0 "	10	5	26
	25.0 "	10	10	19
	30.0 "	10	10	17-35

* All animals were injected subcutaneously with 0.5 mg morphine sulfate 10 minutes before and showed typical symptoms of morphine poisoning.

TABLE II.

Doses of Various Alkaloids Influencing the Effect of Morphine Poisoning on Mice.

Drug	Dose in γ per 20 g mouse*	Effect on morphine excitement and tail phenomenon
Quinine methochloride	100	partial
" ethochloride	500	no effect
" sulfate	500	" "
Tetramethylammonium chloride	100	" "
	200	complete effect
Tetraethylammonium "	114	partial effect
Cryst. thiamine hydrochloride	500	no effect
	1000	complete effect
Acetylcholine "	50	no effect
Histamine "	300	" "
	400	complete toxic effect
Nicotine base	75	no effect
	100	partial toxic effect
Guanidine hydrochloride	1000	no effect
Bulbocapnine "	100	partial effect
	250	partial effect, quiet, tails erect
Scopolamine hydrobromide	100	no effect
	250	partial effect (excited)
	500	complete effect

* All animals were injected subcutaneously with 0.5 mg morphine sulfate 10 minutes before and showed typical symptoms of morphine poisoning.

for Strychnos Curare (Merck), 24.0 ± 2.0 μg; for dihydro-β-erythroidine bromide, 44.0 ± 3.0 μg; and for Intocostrin (Squibb), 20.0 ± 2.0 milliunits.

Several other drugs have been tested for their ability to antagonize morphine excitement and tail phenomenon. These results are shown in Table II. None of these drugs completely antagonize the effects of morphine poisoning in mice in doses smaller than 100 γ per 20 g mouse. Thiamine hydrochloride, quinine ethochloride, quinine sulfate, histamine hydrochloride, bulbocapnine hydrochloride, scopolamine hydrobromide, and guanidine

hydrochloride were without effect in doses of 400-1000 γ. Nicotine base exerted a partial effect in 100 γ dose; histamine was effective only in toxic amounts of 400 γ and acetylcholine chloride was ineffective in a dose of 50 γ. This analysis shows that although the several quarternary alkaloids and other drugs mentioned in Table II may influence the synaptic transmission or the myoneural junction, they do not compare with curare and erythroidine in their influence on the outward manifestion of morphine poisoning in mice. It may be noted here that Myanesin[9] [α,β-dihydroxy-γ-2-(methylphenoxy-propane)] in doses of 8 mg per mouse despite its curare-like action on the striated muscles, is not able to depress the tail reflex in morphinized mice. The data seem to indicate that because of the relatively small amounts of alkaloid required to produce a positive effect, this method may be useful for the determination of curare or erythroidine in tissue extracts or urine even though other active substances may be present.

Summary. A biological method for qualitative and quantitative determination of curare and erythroidine is described. The method depends upon the antagonistic action of these alkaloids on the excitement and tail phenomenon in morphine-poisoned mice. The median effective dose and standard error for crystalline *d*-tubocurarine chloride is 2.8 ± 0.2 μg; for Strychnos Curare (Merck), 24.0 ± 2.0 μg; for dihydro-β-erythroidine bromide, 44.0 ± 3.0 μg; and for Intocostrin (Squibb), 20.0 ± 2.0 milliunits. Far higher doses of other drugs, including quarternary alkaloids, are necessary to antagonize the effect of small amounts of morphine in mice.

[9] Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, **1**, 265.

16295

Effect of Anoxic Anoxia on Propulsive Motility of the Small Intestine.*

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It has been shown that although anoxic anoxia does not significantly affect the propulsive motility of the small intestine of the dog, it does so in the mouse.[1] Since there is this apparent specific difference it was deemed worthwhile to study the effect of anoxic anoxia on still a different species, namely, the rat.

Methods. Paired albino rats as nearly alike in weight and age as possible were fasted 24 hours. One served as a control and the other was subjected to anoxic anoxia. (In all, 42 control and 42 experimental animals were used.) The following partial pressures of oxygen were employed: 94 mm Hg, 80 mm Hg, 63 mm Hg, and 53 mm Hg; corresponding to simulated altitudes of 14,000, 18,000, 24,000, and 28,000 feet.

The experimental animal was given 2 cc of a charcoal-acacia mixture by stomach tube, and after allowing 10 minutes for some of this mixture to enter the small intestine, placed into a low-pressure chamber for 30 minutes. On removal from the chamber it was decapitated and the small intestine removed. The latter was slit open and the distance the charcoal-mixture had traversed the small intestine was measured. Control intubated animals were maintained at atmospheric pressure; in order to keep other experimental conditions as nearly alike as possible, they were placed in cages on top of the low-pressure chamber, so that they were exposed to the same noise and vibration as the experi-

* Aided by a grant of the Ella Sachs Plotz Foundation.

[1] Van Liere, E. J., Northup, D. W., Stickney, J. C., and Emerson, G. A., *Am. J. Physiol.*, 1943, **140**, 119.

TABLE I.
Effect of Anoxia on Peristalsis of the Small Intestine in Rats.

PO_2 mm Hg	Altitude, feet	Control		Anoxia		
		No. of animals	% of gut traversed at end of 40 min.	No. of animals	% of gut traversed at end of 40 min.	"P"*
94	14,000	11	71	11	64	0.19
80	18,000	9	82	11	64	<0.01
63	24,000	10	76	10	50	<0.001
53	28,000	12	78	10	40	<0.001

* Significant when "P" (according to Fisher) is 0.05 or less.

mental animals.

Results and Discussion. The data, Table I, show that anoxic anoxia did not decrease the propulsive motility of the small intestine significantly at a simulated altitude of 14,000 feet. Above this level, however, the motility was significantly decreased by the anoxia.

The results indicated that the threshold for anoxia on the peristalsis of the small intestine lay between a simulated altitude of 14,000 feet and 18,000 feet. In previous work[1] it was shown that the propulsive motility of the intestine of the mouse was not significantly decreased until a simulated altitude of 18,000 feet was reached. The results obtained with rats, therefore, show fair agreement with those for mice. Since anoxic anoxia stimulates the sympathetic division of the autonomic nervous system it would be expected that intestinal movements would be decreased.

The authors still can offer no adequate explanation for the negative effect of anoxic anoxia on the propulsive motility of the small intestine in the dog. The current experiments suggest that as far as the propulsive motility of the small intestine is concerned the rat is somewhat less resistant to anoxia than the dog. However, it should be pointed out that both of these animals are relatively resistant to anoxic anoxia so that the difference observed by us between the two species probably cannot be attributed entirely to this factor.

Summary. The effect of anoxic anoxia on the propulsive motility of the small intestine of the albino rat was studied at the following partial pressures of oxygen: 94 mm Hg, 80 mm Hg, 63 mm Hg, and 53 mm Hg; corresponding to simulated altitudes of 14,000, 18,000, 24,000, and 28,000 feet. At a simulated altitude of 18,000 feet or higher there was a significant decrease in propulsive motility. The threshold lay between 14,000 and 18,000 feet. The results differ from those obtained in the dog but show fair agreement with those in the mouse.

16296

Protein Level and Pteroylglutamic Acid Intake on Growth Rate and Hemoglobin Level in the Rat.

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In attempts to produce a pteroylglutamic acid (PGA) deficiency in the pig, baby pigs were fed a PGA-low "synthetic" diet containing 30% casein and 2% sulfathalidine. However, no blood dyscrasias were observed.[1] Kornberg, Daft and Sebrell[2] and Daft[3] have shown that limitation of the casein in the

[1] Johnson, B., Connor, James, Marian, F., and Krider, J. L., *J. Animal Science*, 1947, **6**, 486.

[2] Kornberg, Arthur, Daft, Floyd S., and Sebrell, W. H., *Science*, 1946, **103**, 646.

[3] Daft, Floyd S., *Fed. Proc.*, 1947, **6**, 405.

TABLE I.
Ration Fed to Each Group of Rats.*

Ingredient	Groups 1	2	3	4	5	6
Casein, Labco	5	5	10	15	20	30
PGA 0.4 mg/100 g	0	0	0	0	0	0
Glucose	83	83	78	73	68	58
Cod Liver Oil	2	2	2	2	2	2
Salt Mixture 446	4	4	4	4	4	4
Corn Oil	4	4	4	4	4	4
Sulfathalidine	2	2	2	2	2	2

* The following vitamins were added per 100 g basal ration:

	mg
Thiamine	.25
Riboflavin	.50
Pyridoxine	.25
Nicotinic Acid	1.0
Calcium-pantothenate	2.0
Biotin	.01
p-Aminobenzoic Acid	5.0
Inositol	10.0
Choline	100.0
2-methyl-1,4-naphthoquinone	0.1
Vit. A, Vit. D	2 drops Haliver oil/rat/week
α-Tocopherol	1 mg/rat/week

diet of the rat is a significant factor in the production of the blood dyscrasias typical of PGA deficiency. In order to determine what level of protein might be fed and still produce anemia on a PGA-deficient ration containing sulfathalidine, the influence of different casein levels and the effect of PGA intake on anemia production in growing rats were investigated.

Experimental. Six groups of weanling rats, 5 in each group, were fed the rations given in Table I. The rats were fed *ad libitum* and kept on experiment for six weeks. Their growth curves are given in Fig. 1. Starting with the third week on the experiment, blood hemoglobin levels and red blood cell counts were determined weekly for the next 4 weeks. The hemoglobin values are given in Fig. 2. The red blood cell counts at 6 weeks are given in Table II.

Discussion. From this experiment it appears that the anemia produced by a protein deficiency on a sulfathalidine-containing PGA-low ration will respond to either PGA or to protein (Fig. 1 and Table IV). On the other hand, the lack of growth on a 5% casein diet was not affected by the addition of PGA to this diet (Fig. 2 and Table III).

The differences between the average gains of the rats for each group were analyzed statistically and no difference was found be-

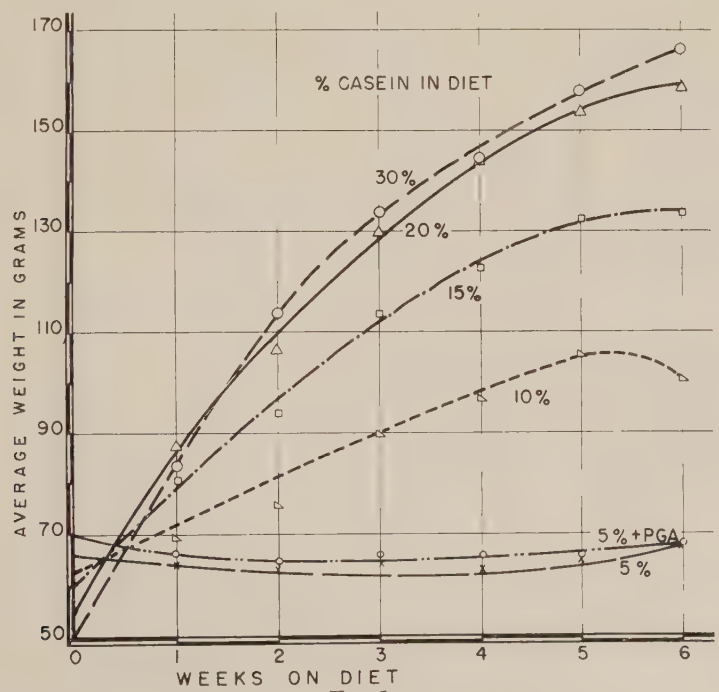

FIG. 1.
Growth curves of experimental rats.

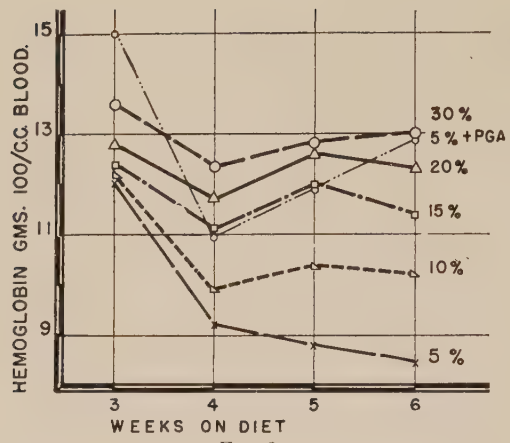

FIG. 2.

Hemoglobin concentration of blood from experimental rats.

TABLE II.

Average Red Blood Cell Count at 6 Weeks for Groups of Rats Fed Increasing Levels of Casein.

Group No.	%	Ration	Avg RBC millions per mm³
1	5	Casein + PGA	7.16
2	5	Casein	5.11
3	10	"	5.95
4	15	"	6.22
5	20	"	6.60
6	30	"	7.15

tween those receiving 20 and 30% protein ($P = 0.45$), but significantly lower rates of gain were found at lower casein levels (30% or 20% versus any other, $P < .01$). The statistical analysis of this data is given in Table III.

Statistical analysis of the data on concentration of hemoglobin in the blood at the end of six weeks, given in Table IV indicated: (1) that the presence of either PGA or of 15, 20, or 30% casein in the diet produced a significantly greater concentration of hemoglobin in the blood than did a 5% casein diet; (2) that diets containing 5% casein plus PGA or 20 or 30% casein produced equal hemoglobin concentrations; and (3) that without PGA, 10% casein produced no significantly greater hemoglobin concentration than did 5% casein. This would indicate that for normal hemoglobin formation in the absence of PGA, over 15% casein is required in the diet, that is, between 15 and 20% casein provides adequate precursors for the synthesis of PGA.

It is also to be noted that even with 2% sulfathalidine in the diet, no significant anemia was observed in the absence of PGA on a 20% casein diet. Several workers[5-9] have produced typical symptoms of PGA deficiency using 18% casein diets with various sulfa drugs used as the intestinal bacteriostatic agents. Our inability to produce blood discrasias at 20% casein may be due to differences in the PGA content of Labco casein, to the inclusion

TABLE III.

Statistical Treatment of Data. Gains of Rats at 6 Weeks (g).

Group No.	1 5% casein + PGA	2 5% casein	3 10% casein	4 15% casein	5 20% casein	6 30% casein
Rat 1	1	8	32	74	126	125
2	—3	2	42	92	95	138
3	—3	1	34	74	108	111
4	—4	—3	52	55	105	104
5	dead	—1	31	82	95	108
Mean	—2.2	1.4	38.2	75.4	105.8	117.2
Rations compared Probability of Difference*		1 vs. 2 .45 1 vs. 3 <.01 1 vs. 4 <.01 1 vs. 5 <.01 1 vs. 6 <.01	2 vs. 3 <.01 2 vs. 4 <.01 2 vs. 5 <.01 2 vs. 6 <.01	3 vs. 4 <.01 3 vs. 5 <.01 3 vs. 6 <.01	4 vs. 5 <.01 4 vs. 6 <.01	5 vs. 6 .45

* Student.[4]

TABLE IV.
Statistical Treatment of Data. Hemoglobin Levels in 100 g/100 cc of Rats at 6 Weeks.

Group No.	1 5% + PAGA casein	2 5% casein	3 10% casein	4 15% casein	5 20% casein	6 30% casein
Rat 2	13.1	8.0	8.3	12.0	12.0	12.4
3	12.4	8.3	11.0	10.7	11.2	14.2
4	13.8	8.9	11.2	10.6	12.4	13.1
5	12.7	8.9	11.0	12.4	13.8	12.4
Mean	13.0	8.5	10.4	11.4	12.4	13.0
Group compared		1 vs. 2	2 vs. 3	3 vs. 4	4 vs. 5	5 vs. 6
Probability of Difference*		<.01	.025	.15	.12	.2
		1 vs. 3	2 vs. 4	3 vs. 5	4 vs. 6	
		<.01	<.01	.025	.025	
		1 vs. 4	2 vs. 5	3 vs. 6		
		.01	<.01	.01		
		1 vs. 5	2 vs. 6			
		.15	<.01			
		1 vs. 6				
		.028				

* Student.[4]

of a more complete list of vitamins (including para-aminobenzoic acid, inositol, etc.) in our ration, to differences in carbohydrate, etc.; or it may be that sulfathalidine is not as useful for prevention of intestinal synthesis of PGA in nutritional experiments as are some of the other insoluble sulfa drugs. However, Teply, Krehl, and Elvehjem[10] have produced growth depression on a 15% casein "synthetic" diet containing 78% sucrose and 2% sulfathalidine, which was partially corrected by feeding a "folic acid" concentrate. Wright, Skeggs, and Sprague,[11] using diets containing 5% sulfasuxidine, have shown that on a high protein (30% casein) diet, "folic acid" stores in the liver and "folic acid" excretion in the feces are higher than on a normal (18% casein) diet. They also obtained better growth on the high protein diet, due to the lower PGA requirement.

Summary. Anemia was produced in growing rats by feeding a ration containing 5% casein and 2% sulfathalidine, to which no pteroylglutamic acid was added. This anemia was prevented either by the addition of pteroylglutamic acid to the diet or by increasing the casein to 20%.

There was no improvement in growth when PGA was added to the 5% casein diet, while 30% casein gave normal growth.

The sulfathalidine used in this experiment was very generously supplied by Sharp and Dohme, Philadelphia, Pa., through the courtesy of Dr. S. F. Scheidy.

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6 Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, **98**, 20.

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16297

Effect of Vagotomy on Gastric Secretory Response to Histamine.*

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During the course of routine studies on the gastric physiology of ulcer patients, it was noted that the gastric secretory response to a standard dose of histamine was decidedly less after complete gastric vagotomy than had been secured in the preoperative period. This surprising finding, which suggested that the physiological action of histamine was not solely upon the cellular elements in the gastric mucosa, prompted the present investigation. Early in the work on histamine, it was reported by Popielski[1] and by Keeton, Luckhardt, and Koch[2] that this drug could produce a gastric secretory response after section and subsequent degeneration of the vagus nerves to the stomach. Ivy and Javois[3] found that histamine would stimulate gastric secretion in a denervated gastric pouch prepared after the method of Bickel, and Klein[4] reported that a similar response could be obtained from a transplanted stomach pouch that had been deprived of its muscular layers and the myenteric plexus. These findings indicate that histamine can stimulate the peripheral neuroglandular mechanism of the stomach, or possibly the gland cell itself. Most investigators agree that histamine acts more intensely on acid producing cells than on the cells which elaborate pepsin. It has been suggested that the increased gastric secretion produced by histamine is secondary to the vaso-dilatation and increased blood supply to the mucosa produced by the drug. A central action of histamine is not indicated. The material presented in this paper includes studies both on experimental animals and man.

Experimental Data. The stomach of dogs was isolated from continuity with the alimentary tract by the method described by Dragstedt and Ellis.[5] Great care was taken not to interfere with the vagus nerve supply and blood supply to the isolated stomach. A gold-plated cannula was introduced for the collection of gastric secretions and the continuity of the alimentary tract was restored by anastomosing the lower end of the esophagus to the upper end of the duodenum. Animals prepared in this fashion were found to secrete large volumes of gastric juice daily and they were kept in electrolyte and fluid balance by the injection of large amounts of salt solution intravenously. When they had entirely recovered from the surgical procedure, which usually required approximately two weeks, they were used for the following studies. The isolated stomach preparation of this type permits the quantitative collection of the gastric secretion from the entire stomach for any desirable period of time. After a control collection of the gastric secretion, one milligram of histamine phosphate was injected subcutaneously and the gastric juice secreted in the following 75 minutes examined for volume, free hydrochloric and total acid secretion. Usually a series of 4 such tests were performed on each animal to determine the average secretory response to histamine. A transthoracic section of the vagus nerves to the stomach was then performed under ether anesthesia with a positive pressure apparatus. The completeness of the vagus section was confirmed in each case by a negative response to insulin hypoglycemia. Following recovery from the vagus section, the animals were tested with histamine exactly as before the operation. The results obtained are indicated in Table I. It is apparent that the volume

* This work has been aided by a grant from the Douglas Smith Foundation for Medical Research, University of Chicago.

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2 Keeton, R. W., Luckhardt, A. B., and Koch, F. C., *Am. J. Physiol.*, 1920, **51**, 469.

3 Ivy, A. C., and Javois, A. J., *Am. J. Physiol.*, 1924, **71**, 604.

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TABLE I.
Effect of Vagotomy on Gastric Secretory Response to Histamine in Total Pouch Dogs.

	Gastric juice secreted in 75 min. after the subcutaneous injection of 1 mg of histamine phosphate				
	Before vagotomy		After vagotomy		
Dog No.	Vol., cc	Total HCl output m. eq.	Vol., cc	Total HCl output m. eq.	Decrease, %
865	162		81		50
		20.7		7.9	62
875	139		45		67
		17.8		4.1	77
898	78		44		44
		8.72		3.42	60

Note: These data present the average of at least 4 tests on each dog.

TABLE II.
Effect of Atropine on Gastric Secretory Response to Histamine Before and After Vagotomy in Total Pouch Dogs.

		Before vagotomy		After vagotomy	
Dog No.	Stimulus	Vol., cc	HCl output, m. eq.	Vol., cc	HCl output, m. eq.
865	1 mg histamine	162	20.7	81	7.9
	2 mg atropine, 30 min. later 1 mg histamine	20	1.4	17	1.2
875	1 mg histamine	139	17.8	45	4.1
	2 mg atropine, 30 min. later 1 mg histamine	24	2.1	19	1.4

Note: These data present the average of at least 2 tests before and after atropine administration in each dog.

of gastric juice secreted in response to the subcutaneous injection of a standard dose of histamine was decreased by 50, 67, and 44% respectively, by complete division of the vagus nerves to the stomach. The total hydrochloric acid output in the gastric juice in response to histamine was diminished after vagotomy by 62, 77, and 60% respectively, as compared with preoperative values. Similar results were obtained in 2 additional animals prepared in the same way. In 2 of the animals, the effect of atropine on the secretion of gastric juice produced by the subcutaneous injection of histamine was determined both before and after section of the vagus nerves to the stomach. The results are indicated in Table II. One milligram of histamine phosphate was injected subcutaneously and the gastric secretion collected for a period of 75 minutes. Two milligrams of atropine sulphate were then given subcutaneously, and 30 minutes later, a second subcutaneous injection of one milligram of histamine phosphate was made. Following the administration of the atropine, the volume of gastric juice secreted in response to the injection of the standard dose of histamine was reduced in the one case by 88, and in the other by 82%. Likewise, the total acid output decreased 93 and 88% respectively. It is interesting that following vagotomy, atropine exerted as great an inhibition to the gastric secretory response to histamine as before vagus section.

Clinical Studies. The studies on man were carried out on 2 groups of patients. The first comprised 15 patients with peptic ulcer who were treated by transthoracic section of the vagus nerves to the stomach at the Albert Merritt Billings Hospital, while the second group included 18 ulcer patients who underwent transabdominal gastric vagotomy at the Illinois State Penitentiary at Stateville. None of the patients in either group had an associated gastroenterostomy. In the first group, the observations were made as follows: Continuous aspiration of the gastric content was made during the night by means of an indwell-

TABLE III.
Effect of Vagotomy on Gastric Secretory Response to Histamine in Patients with Peptic Ulcer.

	Pre-vagotomy		Post-vagotomy			
Patient	Vol., cc	Total acid output, m. eq.	Vol., cc	Total acid output, m. eq.	Reduction in vol., %	Reduction in acid output, %
No. 24	168	10.1	103	5.9	39	41
68	170	10.9	101	2.0	41	81
73	272	28.9	200	11.2	26	61
71	198	19.0	203	12.8	—3	33
74	338	40.6	151	13.6	56	66
17	292	18.8	95	6.5	67	65
22	164	11.6	84	2.7	49	76
23	194	10.3	109	5.2	44	49
24	168	11.0	186	5.5	—1	50
26	353	39.8	131	5.5	63	86
27	192	21.8	130	12.8	32	41
31	143	4.8	68	2.6	53	46
32	63	2.6	45	.8	30	69
34	243	25.6	43	2.5	82	90
35	81	4.0	52	1.1	36	72
Avg	203	17.3	113	6.1	41	62

Note: One mg of histamine phosphate was given subcutaneously in the fasting state and the gastric content removed by continuous suction for 60 minutes.

TABLE IV.
Effect of Vagotomy on Gastric Secretory Response to Histamine in Male Prisoners with Peptic Ulcer.

	Pre-vagotomy		Post-vagotomy			
Patient	Vol., cc	Total acid output, m. eq.	Vol., cc	Total acid output, m. eq.	Reduction in vol., %	Reduction in acid output, %
No. 10	176	22.2	154	16.8	13	24
4	267	28.8	124	11.9	53	59
11	315	42.5	98	3.7	68	91
8	274	30.7	32	3.0	88	90
9	262	31.4	84	4.7	67	85
5	315	27.1	162	15.1	48	44
14	200	20.6	84	3.1	58	85
13	290	33.3	13	.2	95	99
15	210	26.0	180	17.1	14	34
19	232	24.1	28	.4	88	97
22	350	41.6	88	1.8	75	95
21	286	28.6	80	.2	72	99
25	118	8.1	94	6.3	12	22
28	142	13.4	180	9.7	—21	29
26	210	25.6	40	.7	81	97
24	166	15.8	58	1.2	65	92
30	308	40.9	88	1.9	71	95
23	238	25.9	94	.5	60	97
Avg	242	27.0	94	5.5	56	74

ing gastric tube and the Wangensteen suction apparatus. In the morning, one milligram of histamine phosphate was injected subcutaneously and the gastric content aspirated at 10-minute intervals. Similar tests were made from 7 to 10 days after the gastric vagotomy. The volume of gastric juice secreted during a period of 60 minutes was measured and its free acid concentration determined. From these figures, the total acid output was calculated for each test. In the second group of patients studied at the Illinois State Peniten-

tiary, the tests were performed in a similar manner, except that the gastric content was collected by continuous Wangensteen suction for a period of 60 minutes after the subcutaneous injection of the standard dose of histamine. The postoperative tests were performed from 3 to 6 weeks following the gastric vagotomy when the patients had entirely recovered from the operation. The effect of gastric vagotomy on the secretory response to histamine in the 15 ulcer patients studied at the University of Chicago Clinics is illustrated in Table III. In this series of patients, the vagotomy produced a decrease of 41% in the volume of gastric juice secreted in response to the histamine injection and a decrease of 62% in the total amount of hydrochloric acid produced. The free acid concentration for the 15 tests decreased from an average of 93 clinical units to 67 following the vagus section. Table IV illustrates the results secured in the 18 patients with peptic ulcer studied at the Illinois State Penitentiary. In these patients, the average volume of gastric juice secreted in response to a standard dose of histamine decreased 56%, and the total acid output 74% after the vagotomy. The average free acid concentration of the histamine stimulated gastric juice averaged 110 clinical units before vagotomy and 48 clinical units after the operation.

Conclusions. 1. The gastric secretory response to a standard dose of histamine in dogs with a totally isolated stomach pouch is markedly reduced by bilateral vagotomy.

2. Atropine similarly reduces the gastric secretory response to histamine in these animals.

3. Gastric vagotomy in patients with peptic ulcer also markedly reduces the gastric secretory response to a standard dose of histamine.

16298 P

Effect of Bile Preparations on Fat Absorption in Bile Fistula Dogs.*

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That bile is concerned in the absorption of dietary fat has been qualitatively established by the observation of steatorrhea in animals[1] and in man[2] when bile fails to reach the intestine. The effectiveness of human whole bile in correcting this absorptive defect has been shown in only 2 patients,[3] and bile acid preparations have not been tested directly in man or experimental animals. Inconclusive and contradictory results from studies of blood fat after a fatty meal,[4] absorption from isolated intestinal loops,[5,6] and *in vitro* diffusion[7] or solubilization[8] constitute the basis for the belief that bile acids are essential for fat absorption, and that

* Supported in part by a grant from G. D. Searle and Company.

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TABLE I.
Fecal Fat Excretion in Bile Fistula Dogs.

Regime	No. dogs compared	Means of changes from fecal fat output of 27.4 g/day on no-bile regime, g fat/day	Each regime compared with no-bile, t-ratio
3 g dehydrocholic acid	8	+ 1.61	0.71
3 " iron ppt'd ox bile*	7	— 3.20	1.61
3 " desoxycholic acid	5	+ 0.14	0.06
3 " desiccated ox bile†	7	— 1.47	0.72
6 " " " "	6	— 7.50	3.58‡
90 cc " " "	7	—14.90	7.60§

* Contains 47% cholic and 48% desoxycholic acids.
† Contains about 50% cholic acid and undetermined desoxycholic acid.
‡ Significant at 5% level.
§ Significant at 1% level.

desoxycholic acid is particularly active. In view of the inadequacy of the indirect, and the lack of direct evidence, studies were undertaken to evaluate the relative effectiveness of various bile acids in restoring normal fat absorption in bile fistula dogs.

Methods. Fecal fat excretion was determined in 9 dogs before and after cholecystonephrostomy, in the latter with and without administration of various bile preparations. The daily diet consisted of 335 g "Pard" with 25 g added lard (36 g total fat). Food was given once daily, and was completely eaten. Each regime consisted of a 7-day period; during the last 5 days feces were pooled and analyzed for total fat.[9] When given, bile preparations were mixed with the meal (pills, capsules, or fresh bile). Following operation the feces were free of urobilinogen. Neither jaundice nor diarrhea developed, although stools were bulky.

The plan to subject each dog to each regime was not completed because 3 dogs developed duodenal ulcers and 2 dogs did not tolerate 3 g doses of desoxycholic acid. Statistical analyses were made by comparing 2 regimes in the same animal.

Results. The mean fecal fat excretion of the 9 dogs was increased from 3.0 g/day before operation to 27.4 g/day after diversion of bile from the intestine. Three-gram daily doses of bile acids were given in an effort to correct this steatorrhea. This dose is the maximum that is tolerated for some of the preparations, and is also the 8-hr output of cholic acid by healthy bile fistula dogs when their bile is returned every 8 hours.[10] The failure of iron-precipitated ox-bile (Bilron, Lilly) and desoxycholic acid (Degalol, Ames) to reduce fat excretion was wholly unexpected. Dehydrocholic acid (Ketochol, Searle) was expected to have less effect than the other products tested (Table I). On the basis that the above bile acids may have been altered in their purification, desiccated ox bile and fresh ox bile (kept frozen until use) were administered. Three-gram doses of desiccated ox bile were ineffective; 6-g doses produced a slight but significant reduction in fecal fat. Ninety-cc doses of fresh ox bile, equivalent to 6 g desiccated material, produced a significantly greater, but still incomplete improvement in fat absorption.

Discussion. These results indicate that 3-g doses of certain bile preparations are completely ineffective in replacing the fat-absorptive function of normal whole bile in the dog. It appears that desiccation reduces the ability of fresh ox-bile to promote fat absorption. These results agree with an earlier report[6] that fresh bile, but not sodium glycocholate or taurocholate, can facilitate the disappearance of sodium oleate from isolated intestinal loops.

No convincing explanation can be offered for these results at present. They are con-

[9] Fowweather, F. S., and Anderson, W. N., *Biochem. J.*, 1946, **40**, 350.

[10] Berman, A. L., Snapp, E., Ivy, A. C., and Atkinson, A. J., *Am. J. Physiol.*, 1941, **131**, 776.

trary to general beliefs concerning the role of bile acids in fat absorption, but many speculative possibilities await study. Further direct experimentation is required to determine (a) whether the absorptive defect consequent to bile exclusion can be completely corrected, (b) the identity of the active constituent(s) in bile (c) the effect, if any, of the manner of entry of active material into the intestine, (d) the role, if any, of species differences, and (e) the mechanism by which fat absorption is facilitated.

Summary. 1. Three-gram doses of certain bile preparations (including desoxycholic acid) proved completely ineffective in reducing the steatorrhea of bile fistula dogs.

2. Ninety-cc doses of fresh ox bile reduced the steatorrhea by 50%, but equivalent doses (6 g) of desiccated ox bile were only half as effective.

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Increased Hemolytic Potency of Mouse Mammary Carcinoma Extracts Following Incubation with Tumor Cells.*

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It was recently observed in this laboratory that filtered or centrifugated extracts prepared from spontaneous mouse mammary carcinomas possess the ability to hemolyze mouse erythrocytes *in vitro*.[1]

In course of subsequent experiments it was found that tumor extracts that had been separated from the tumor cells by centrifugation, and have been left, in absence of cells, in test tubes, at room temperature, lost practically all hemolytic potency after 5 hours; the decrease in the hemolytic potency of the tumor extracts was even more rapid at 37°C.[2] This rather striking lack of resistance of the hemolytic factor contained in the tumor extracts, but separated from the tumor cells, to exposure for a few hours to either room, or incubator temperature, was further investigated. One of the first questions requiring clarification was whether the tumor extracts would also lose their hemolytic potency if incubated, at 37°C, in presence of tumor cells.

Accordingly, a series of experiments was performed in which tumor cell suspensions of 20% concentration were freshly prepared from spontaneous mouse (C3H) mammary carcinomas.[2] Fresh samples of such suspensions were tested for their hemolytic activity.[1] The suspensions were immediately divided into 2 groups of tubes: one was left unchanged as a control, and the other was centrifugated twice at 3,000 t.p.m. for 10 minutes each, to separate the extracts from the tumor cells; following centrifugation, the final supernatant fluid was used. Both, the tumor cell suspensions, and the cell-free extracts, were then placed in an incubator, at 37°C, for 5, and 24 hours. After incubation, samples removed from both groups of tubes were tested for their hemolytic activity by serial titration. Eight different mouse mammary carcinomas were used for the preparation of extracts, and 8 successive series of experiments were performed under apparently identical conditions. The results were as follows:

All 8 fresh tumor extracts were found to possess the usual hemolytic potency[1] when tested before exposure to the incubator temperature. After exposure for 5, or 24 hours, to 37°C, the centrifugated tumor extracts incubated without cells, were found to have lost their hemolytic potency, when tested in

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

1 Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 292.

2 Gross, L., *J. Immunol.*, 1948, in press.

the usual[1] manner (0.5 cc of an extract of 20% concentration). On the other hand, the same tumor extracts incubated in presence of tumor cells, at 37°C, for either 5 or 24 hours, had not only their hemolytic potency preserved, but either moderately or substantially increased, as compared with the initial titration of fresh extracts. Thus, one of the fresh extracts was found to hemolyze mouse erythrocytes only when tested (0.5 cc) in 20% concentration, *i.e.*, at 1:5. Following incubation in presence of tumor cells at 37°C for 5 hours, the same extract (0.5 cc) hemolyzed readily mouse erythrocytes in dilutions up to 1:40. After an additional incubation with tumor cells at 37°C to a total of 24 hours, this extract hemolyzed mouse erythrocytes in dilutions up to 1:160.

In some instances the increase in hemolytic potency appeared to be slightly more pronounced after 5 than after 24 hours of incubation with tumor cells. In one experiment an extract which had been found to hemolyze mouse erythrocytes, when tested prior to incubation, only at either 1:5, or 1:10, had after 5 hours of incubation at 37°C with tumor cells its hemolytic potency increased to such an extent that serial dilutions up to 1:160 gave positive readings. After an additional incubation with tumor cells to a total of 24 hours, however, the hemolytic potency of the same tumor extract was slightly decreased, giving positive readings in dilutions not exceeding 1:40; thus, it was still higher than that of the initial titration of the fresh tumor extract, but less pronounced than that tested after 5 hours of incubation.

Control Experiments with Normal Cells. It was previously observed[1,2] that fresh extracts prepared from normal mouse organs, such as liver, spleen, kidneys, muscle, or lungs, had no hemolytic potency on mouse erythrocytes *in vitro* in practically all instances tested,† except for extracts made from active mammary glands removed from nursing or pregnant female mice of either a high-tumor, or a low-tumor line.[2]

In the present study additional experiments were performed in which normal mouse cell suspensions were incubated at 37°C for either 5 or 24 hours, and then tested for hemolytic potency. Accordingly, normal organs, such as liver, spleen, kidneys, muscle, and lungs, were removed from a total of 12 healthy, adult, virgin female mice of the An (C3H)[3] subline, and either individual or pooled cell suspensions of 20% concentration were prepared from each mouse. Fresh samples of the extracts were tested for hemolytic activity.[1] The cell suspensions were incubated at 37°C for either 5 or 24 hours; after this lapse of time, the suspensions were centrifugated, and the supernatant extracts were tested for hemolytic potency in the usual manner.[1] The results were as follows:

Fresh samples of the normal cell extracts had no hemolytic potency.† This result was essentially consistent with previous observations.[1,2]

With the extracts obtained from normal cell suspensions that had been *incubated for 5 hours*, the following readings were obtained: Of 8 individual liver extracts tested (0.5 cc), 1 was positive in 1:10 dilution. Of 8 pooled (kidney, spleen, muscle, lungs) extracts tested (0.5 cc), 3 were positive in 1:10 dilution. All other extracts, including 4 groups of cell suspensions made from individual organs (lungs, spleen, kidneys, muscle) of 4 females, were negative.

A few tests were made with extracts obtained from cell suspensions that had been *incubated for 24 hours*. Two of 4 liver extracts, and 2 of 4 pooled extracts were positive (0.5 cc) at 1:10. One of 3 lung extracts tested was positive at 1:5, and 1 of 3 muscle extracts at 1:5, and 1:10. All other extracts, including 3 spleen, and 3 kidney extracts, were negative.

Summary. If separated from the tumor cells by centrifugation, the mouse mammary

† This was true under routine conditions of the test, *i.e.*, incubation of the extracts with mouse erythrocytes at 37°C for 2½ to 3 hours.[1] It was found, however, that normal mouse organs may occasionally show a "delayed" hemolytic potency; thus, when extracts prepared from normal mouse cells were mixed with mouse erythrocytes, and incubated first at 37°C for 5 hours and then at 7°C (refrigerator) for additional 15 hours, a slight hemolysis resulted in some instances.

[3] Gross, L., *J. Immunol.*, 1947, **55**, 297.

carcinoma extracts lose their hemolytic potency, when incubated at 37°C, after 5 hours. On the other hand, the hemolytic potency of the tumor extracts is either moderately, or substantially increased following incubation in presence of tumor cells, at 37°C, for either 5 or 24 hours.

Fresh extracts prepared from normal mouse cells have in most instances no hemolytic potency, except for those made from active mouse mammary gland.[2] If the normal mouse cell suspensions (liver, lungs, muscle, or pooled organs), however, are incubated at 37°C for 5, or better for 24 hours, some of them may show a slight hemolytic potency, though to a lesser degree than that observed in the case of pre-incubated tumor cell suspensions.

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Reconstitution of the Dermal Barrier to Fluid Diffusion Following Administration of Hyaluronidase.*

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It is now generally accepted that hyaluronidase owes its spreading activity to the action of the enzyme upon a viscous hyaluronic acid gel in connective tissues[1,2] which is one of the components of the tissue barrier to interstitial fluid diffusion.[3] The effect of hyaluronidase in facilitating the spreading in skin remains evident in the locally treated area for as long as 24 hours after administration of the enzyme, and thereafter is absent.[4] This demonstrates that after a certain latent period, the organism replaces the component of the skin barrier removed by the hyaluronidase.

It appeared likely that a quantitative study of the rate at which the dermal barrier to spreading of fluids was restored, after administration of hyaluronidase, might provide information concerning the formation of new dermal hyaluronic acid *in vivo*. Accordingly, such a study was undertaken in adult humans.

The hyaluronidase used was a purified bovine testis preparation (Schering) which assayed 20 turbidity-reducing units (TRU) per mg as evaluated by the method of Kass and Seastone.[5] The enzyme was used directly after dissolution in 0.85% sodium chloride. Three men and 3 women received intradermal injections of 0.20 cc of enzyme solution containing 20, 2, 0.2, 0.02, 0.002, and 0.0 TRU per cc into separate areas of the 2 arms. After the injection of the enzyme solutions, the local areas were marked with tincture of merthiolate. The time of wheal disappearance following intradermal injection served as a measure of hyaluronidase spreading activity. The local area was examined continuously for 10 minutes after the injections, then at 5-minute intervals until the wheal has disappeared, *i.e.*, until the injected local area appeared completely flat as viewed from a 90° angle. At 24, and then again at 48, hours after the initial injections of enzyme 0.20 cc of an 0.85% solution of NaCl was injected into each previously treated area, and the time of wheal disappearance determined.

Table I illustrates the interrelationships between the time required for wheal disappearance and the concentration of enzyme injected. On the assumption that the time of wheal disappearance is a measure of the barrier efficiency of the dermal hyaluronic acid gel associated with the tissue barrier to

* Aided by a grant from the G. D. Searle & Co.

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2 Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

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TABLE I.
The Wheal Disappearance Time of Intradermally Administered Saline, Injected into Areas Treated with Hyaluronidase.

		Time of saline injection after hyaluronidase				
		0 hr	24 hr		48 hr	
No. of subjects	Amt of hyaluronidase,* T.R.U.	W.D.† min.	W.D. min.	B§ %	W.D.† min.	B§ %
6	4	0.20 ± 0.06‡	6.50 ± 1.8	11	62.5 ± 6.6	100
6	4×10^{-1}	0.78 ± 0.17	13.7 ± 2.8	23	61.7 ± 7.2	100
6	4×10^{-2}	3.0 ± 0.70	24.7 ± 4.3	41	61.7 ± 8.5	100
6	4×10^{-3}	6.1 ± 1.2	36.7 ± 4.7	61	67.5 ± 5.9	100
6	4×10^{-4}	15.0 ± 5.6	46.7 ± 6.2	78	63.3 ± 6.6	100
6	0	65.0 ± 8.4	58.0 ± 9.0	—	61.1 ± 7.8	—

* Intradermal administration in a constant volume of 0.20 cc.
† W.D. is the mean time of wheal disappearance.
‡ Is the standard error of the mean.
§ B is the percentage of the normal effective barrier present after enzyme administration assuming (a) a linear relationship between effective barrier and spreading and (b) that a normal barrier has a wheal disappearance time of 60 minutes.

diffusion, the percentage restoration of dermal hyaluronic acid 24 and 48 hours after the administration of enzyme has been calculated and these values recorded in Table I.

Twenty-four hours after the administration of hyaluronidase in amounts ranging from $4 \times 4 \times 10^{-4}$ TRU (in a volume of 0.2 cc), the dermal barrier of the local area of injection exhibited a permeability which was directly relateed to the dose of the enzyme; after 48 hours, the permeability of the barrier in all treated areas was decreased to normal.

In skin, purified testis hyaluronidase appears to act specifically upon hyaluronic acid[2] and does not affect other mucopolysaccharides, such as chondroitin sulfate, which is present in a concentration equivalent to that of hyaluronate.[2] This does not hold for other tissues, purified testis hyaluronidase having been reported to hydrolyze the monosulfuric acid ester of hyaluronic acid obtained from cornea[6] and the chondroitin sulfate of hyaline cartilage.[7,8] We have found that injection of hyaluronidase into areas which have "recovered" from a previous dose of enzyme produces its usual spreading reaction, thus strongly suggesting that new dermal hyaluronate, and not another mucopolysaccharide, insensitive to hyaluronidase, is involved in the reconstitution of the barrier.

Summary. The reconstitution of the dermal barrier removed by intradermal injection of various doses of hyaluronidase has been studied 24 and 48 hours after enzyme injection in adult humans. At 24 hours the restoration of the barrier is incomplete and inversely related to the dosage of enzyme; at 48 hours the barrier is completely restored in all enzyme-treated areas.

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16301

Hepatic Fibrosis in the Persistently Non-Fatty Liver of the Hypophysectomized Dog.

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The frequent association of hepatic lipoidosis and necrosis with cirrhosis in man and experimental animals has led to the view that fatty and other forms of liver-cell degeneration induce cirrhosis. Where fatty changes did not precede hepatic fibrosis, hemorrhagic or some other form of hepatocellular necrosis has usually been considered to be the precipitating lesion.

That some factor or factors other than liver-cell damage may possibly be responsible for the initiation and progress of hepatic fibrosis is brought out in the present investigation. Fibrosis was found in livers of hypophysectomized dogs in which the fatty-acid content never exceeded 4%, *i.e.*, normal concentrations, and in which evidence of extensive liver-cell destruction was not detected histologically.

Experimental Procedures. For the first 3-4 weeks after their arrival at the laboratory the dogs were fed a diet high in protein and adequate in all respects. Each dog received twice daily, at 8:00 a.m. and at 4:00 p.m., 15 g of lean meat per kilo, 10 g of sucrose, 4 g of bone ash, and 1 g of Cowgill's salt mixture.[1] Vitamin supplements were provided by the addition of 3 cc Sardilene† and 5 cc of Galen B.† After the dogs had received this diet for 2-3 weeks, they were hypophysectomized by a method previously described.[3] In the procedure used in this study no cautery was applied to the base of the brain. In most cases the appetites of the dogs were excellent after hypophysectomy.

At the end of the periods of observation recorded in Table I, the whole livers of the dogs were excised while they were under nembutal anesthesia. Several sections of each liver were removed for histological study, and the rest was ground and sampled for lipid analysis as described in a previous paper.[4]

Serial sections were made of the base of the brain of each dog and every fifth section carefully examined. No cells of the anterior, posterior, or intermediate lobes of the pituitary gland were found in the dogs recorded in this study; nor was there any evidence of damage to the hypothalamic region.

Results. The chemical and histological findings in the livers of these animals have been summarized in Table I. In an earlier study the fatty-acid contents of dogs' livers were examined at intervals of 2-4 months after hypophysectomy;[5] no deviation from the normal was found at these time-intervals. The results recorded in Table I leave no doubt that hypophysectomy *per se* does not influence the fat content of the liver, even 32 months after it has been performed.

Histologically, the liver cells did not show any evidence of fatty change or necrosis. The only abnormal findings in the liver cells were firstly an unusual distinctness of the cell walls with clumping of the chondriosomes (plant-like cells), and secondly an eosino-

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1 Cowgill, G. R., *J. Biol. Chem.*, 1923, **56**, 725.

† Each cubic centimeter of Sardilene contained not less than 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A. The vitamin content of Galen B has been recorded elsewhere.[2]

2 Montgomery, M. L., Entenman, C., Chaikoff, I. L., and Nelson, C., *J. Biol. Chem.*, 1941, **137**, 693.

3 Dandy, W. E., and Reichert, F. L., *Bull. Johns Hopkins Hosp.*, 1925, **37**, 1.

4 Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

5 Chaikoff, I. L., Gibbs, G. E., Holtom, G. F., and Reichert, F. L., *Am. J. Physiol.*, 1936, **116**, 543.

TABLE I. Pathological Findings and Fatty Acid Content of Livers of Hypophysectomized Dogs.

Dog	Body wt			Sacrificed after hypophysectomy, mo.	Liver findings					
	Initial, kg	Max., kg	Final, kg		Pathological					
					Fibrosis*	Cirrhosis†	Plant-like cells	Eosinophilic hyalinization	Wt, g	Total fatty acids, %
H25	7.2	10.0	9.7	24	3+	? early	2+	2+	220	2.2
H26	7.6	8.6	8.1	24	0	0	2+	2+	170	1.9
H27	7.2	8.1	7.8	24	patchy slight	0	2+	2+	152	4.9
H28	6.2	8.2	8.2	24	4+	+	2+	+	176	2.7
H29	6.4	10.6	9.1	24	3+	+	2+	+	196	3.9
H32	4.1	8.3	8.3	32	0	0	3+	0	265	3.8
H33	5.5	6.7	6.7	32	0	0	3+	2+	212	3.2

* *Hepatic fibrosis* or precirrhosis indicate the presence of abnormal amounts of fibrous tissue in the liver without architectural distortion of the lobular pattern.

† *Cirrhosis* indicates the supervention of architectural distortion in association with hepatic fibrosis.

Histological grading of "plant-like" cells and eosinophilic hyalinization:

Assessment of degree of fibrosis:

+ = Scattered foci.
2+ = About ¼ of lobule affected.
3+ = About ½ of lobule affected.
+ = Present and in scattered foci.
3+ = About ½ of lobules affected.
4+ = About ¾ of lobules affected.

philic hyalinization of the liver cells. Both these changes were widespread in the livers of *all* the dogs and apparently occurred independently of the incidence of fibrosis. While the eosinophilic hyalinization occurred in all the livers, this reaction was most prominent around the non-fibrosed portal tracts (Fig. 1). Apart from these changes in the liver cells, the majority of livers appeared relatively avascular by virtue of the fact that most of sinusoids were closed and did not contain blood. This occlusion of the sinusoids and the close apposition of the individual liver cells to one another gave the liver a highly cellular appearance (Fig. 1).

The fibrotic reaction to be described below was detected, in varying severity, in 4 of the 7 hypophysectomized dogs. This connective-tissue reaction was most unusual in its form, location, and distribution in the affected livers. In the first place not all the lobes of the same liver were affected by this reaction and, had we not had sections from several parts of each liver, we would clearly have missed the change in at least 2 of the dogs which manifested this lesion. It is possible that the patchy distribution of this lesion was due, in several of our dogs, to the fact that it had begun only a short time before death. It is probable that, had the dogs been maintained for longer periods of time, the fibrosis would have become both more extensive and more severe.

When present, the fibrosis which we detected was most frequent and most severe in and around the portal tracts, although by no means confined to these regions (Fig. 2). The fibrosis took two forms, which possibly represented different stages in the evolution of the lesion. In its most striking form it appeared as a highly cellular connective-tissue proliferation (Fig. 2, 3, and 4). This cellularity was due primarily to the large numbers of fibroblast and fibrocyte nuclei. The portal tracts, both large and small, were made very prominent by the accumulation around them of round cells and numerous closely packed, spindle-shaped, young-fibrous-tissue nuclei (Fig. 2 and 3). Often, when sections were cut obliquely through what, by location, seemed to be portal tracts, sheets of this highly

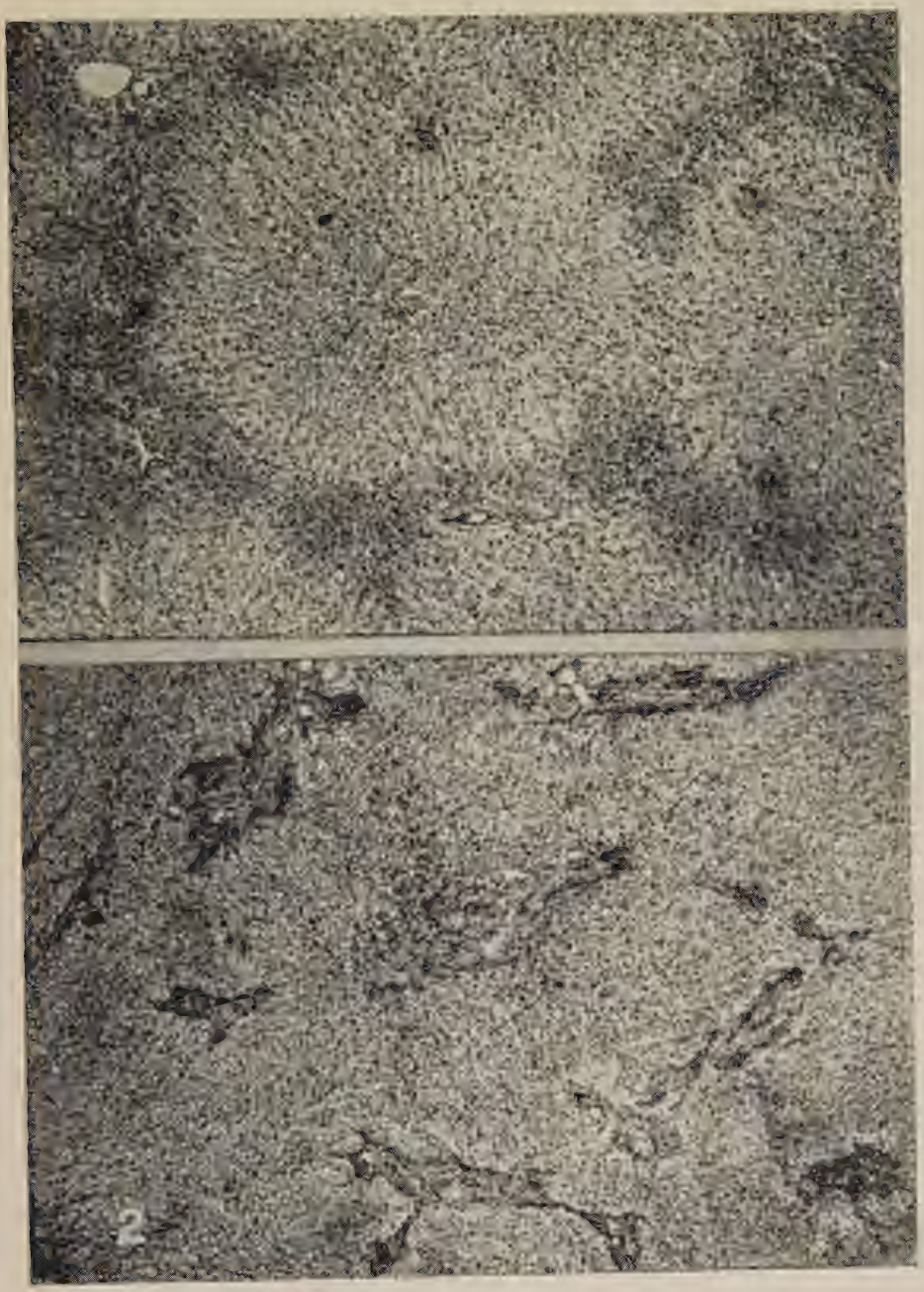

Fig. 1.

(H32) The periportal localization of the eosinophilic hyalinization with nuclear hyperchromasia of the liver cells is clearly shown. Note also "plant-like" cells in the rest of the lobule and the avascularity of the liver. Note also the absence of fatty change. Hematoxylin and Eosin × 45.

Fig. 2.

(H28) The cellular periportal fibrosis and the less highly cellular reaction in the central portion of the lobule are typical of the changes seen in these livers. Hematoxylin and Eosin × 70.

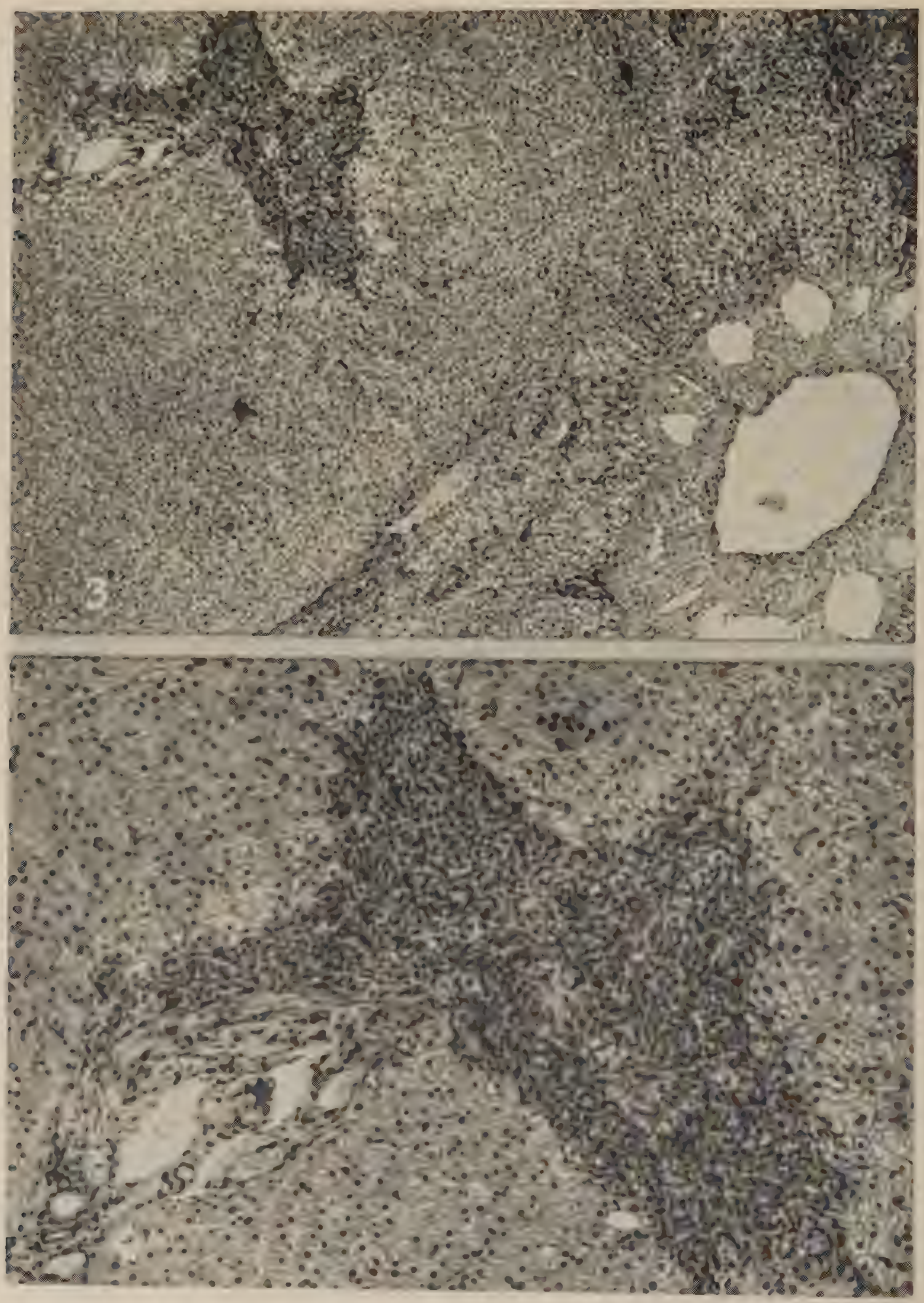

FIG. 3.

(H29) The portal tract towards the top left corner of the picture is made prominent by the marked accumulation of cells. Scattered bands of fibrous tissue can also be detected in this picture. Hematoxylin and Eosin × 64.

FIG. 4.

(H29) Details of the nature of the cells infiltrating the portal tract depicted at the top left corner of Fig. 3. In addition to round cells there are large numbers of spindle-shaped fibrocytic nuclei. Hematoxylin and Eosin × 152.

cellular connective tissue seemed to be stretched across the liver lobules. However, in other areas these sheets of young connective tissue seemed to be related to radicles of the hepatic veins or occurred in the middle of apparently intact liver-cell lobules (Fig. 2). Such masses of young connective tissue occasionally joined together, thus outlining 2 sides of a lobule, or, when more extensive, it obliterated a liver lobule. This latter reaction when present was most common in the subcapsular regions, although in the more severely affected livers such obliteration of liver tissue was more widespread.

The great cellularity of this connective tissue was very similar to that detected by Graef *et al.*[6] in the fatty livers of their hypophysectomized dogs (*cf.* their Fig. 5 and 6 with our Fig. 2 and 3). However, in many of the fibrosing areas the cellularity was made less prominent by the fact that masses of dense, highly eosinophilic collagen fibers had been deposited among the still numerous fibroblast and fibrocyte nuclei (Fig. 2 and 3).

Associated with this fibrous tissue were numerous histiocytic cells containing very dark-brown coarse pigment granules. Similar pigmented histiocytes have been encountered in smaller numbers in the livers of normal dogs, but such pigmented cells were not detected in the non-fibrotic livers of hypophysectomized dogs.

Discussion. The relation of liver-cell damage to the onset of cirrhosis has been much discussed in recent years. Although the connective-tissue changes in response to hypophysectomy are not so pronounced as those encountered in livers in which extensive fatty infiltration or necrosis has been induced, the present findings leave no doubt that the stimulus to connective-tissue proliferation in the liver need not be hepatocellular degeneration, either fatty or necrotic. The greatest amount of fibrous-tissue proliferation occurred in the livers of dogs H25, H28, and H29; in these, fatty change or degeneration of the liver cells was not detected.

The mechanism by which hypophysectomy brings about fibrous-tissue proliferation in the liver is not clear at present. Graef *et al.* stated that "it appears reasonable to conclude that hypophysectomy itself . . . . had little if anything to do with the hepatic lesions encountered in these dogs. The hypothalamic lesions disclosed by histological examination seemed to have a pivotal role in the hepatic changes." Careful histological studies of the hypothalamic regions in *our* animals, using the serial-section technic mentioned above, failed to reveal any evidence of damage to this important center. This fact, together with the absence of fat from the livers of our hypophysectomized dogs, indicates that the pathogenesis and possibly the etiology of the hepatic fibrosis in our animals differed somewhat from those described by Graef *et al.*

The protein content of the diets of our animals was high, and consequently low-protein *intake* could not be blamed as a factor in the etiology of the hepatic fibrosis in them. However, some interference in the absorption process has been shown to follow excision of the pituitary gland.[7,8] Hence despite the ingestion of a high-protein diet by our dogs, it is conceivable that hypophysectomy induced some disturbance in the digestion or absorption of certain dietary substances which play a critical role in maintaining a normal liver. The possibility that hypophysectomy promotes an increased need for such substances is, of course, not ruled out.

Summary. 1. Fibrous-tissue proliferation is shown to occur in the livers of hypophysectomized dogs. Its development was not preceded by an increase in the fat content of the liver.

2. Hypothalamic damage as a cause of the fibrous-tissue proliferation was ruled out.

3. The development of hepatic fibrosis in these dogs fed a diet adequate in all respects and rich in proteins suggests that hypophysectomy induces either (a) a derangement in digestion and/or absorption, or (b) an increased need for certain dietary substances essential for the maintenance of a normal liver.

[6] Graef, I., Negrin, J., and Page, I. H., *Am. J. Path.*, 1944, **20**, 823.

[7] Russell, J. A., *Am. J. Physiol.*, 1938, **121**, 755.

[8] Althausen, T. L., *Essays in Biology in Honor of H. M. Evans*, University of California Press, Berkeley, 1943, p. 13.

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A Quantitative Study of the Effect of Vagotomy on Gastric Secretion in the Dog.*

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Lim, Ivy, and McCarthy[1] described the use of a pouch of the entire stomach in studying the physiology of gastric secretion in the dog. This preparation is sometimes called a Fremont pouch, since Fremont[2] described this procedure in 1895.

In the total gastric pouch used by Lim, Ivy, and McCarthy, the vagus nerves to the stomach were divided when the cardia was transected. These dogs secreted 200 to 300 cc of gastric juice with a relatively low free acidity. Dragstedt and Ellis[3,4] described the total gastric pouch prepared with the vagus nerves intact. Unlike the preparation used by Lim, Ivy, and McCarthy, such an animal secretes 600 to 2,000 cc of highly acid gastric juice daily, and will succumb to hypochloremia and alkalosis in 3 to 10 days unless given daily intravenous infusions of Ringer's solution. These animals develop large penetrating peptic ulcers in the gastric pouch,[5] and frequently die of perforation or hemorrhage.

The present study was undertaken to determine quantitatively the effect of vagotomy on hydrochloric acid and pepsin secretion in the total pouch dog, and to determine the effect of vagotomy on secretion in response to food and other stimuli. A total of 12 dogs survived surgery. Vagotomy was performed in 9 animals by the transthoracic approach.

With the animals on a standard diet, the 24-hour gastric secretion was collected, titrated for free acidity, and the peptic power determined by a method described by LeVeen.[6]

In each animal, the integrity of the vagus nerves to the gastric pouch was established by the use of the intravenous insulin test, as described by Ihre[7] and Jemerin, Hollander, and Weinstein.[8] In one dog, the test was negative, and secretion remained low in volume and acidity. The response to histamine was determined by injecting one milligram subcutaneously and collecting the gastric juice over a 75-minute period. The response to food-taking was measured by feeding the fasting animal a standard meal of meat over a 15- to 30-minute period, and by collecting fractional samples of gastric juice for 12 to 16 hours. The same testing procedures were carried out following vagotomy.

Vagotomy produced a decrease in the volume and free acidity of the 24-hour gastric secretion in all of the 9 animals which were vagotomized. The decrease in volume varied from 37 to 79%, averaging 56%. The decrease in free acidity varied from 14 to 82%, averaging 54%. The decrease in milliequivalents of free hydrochloric acid secreted varied from 47 to 92%, the average reduction in hydrochloric acid output being 77%.

In 7 of 8 animals, vagotomy produced a reduction in the peptic power of the 24-hour secretion. This reduction varied from 11 to 72%, averaging 34%. In one animal, the peptic power increased by 47%.

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1 Lim, R. K. S., Ivy, A. C., and McCarthy, J. E., *Quart. J. Exp. Physiol.*, 1925, **15**, 13.

2 Fremont, *Bull. de l'Acad. de Med.*, 1895, **34**, 509.

3 Dragstedt, L. R., and Ellis, J. C., Proc. Soc. Exp. Biol. and Med., 1929, **26**, 305.

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6 LeVeen, H. H., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 254.

7 Ihre, B. J. E., *Acta Med. Scandinav.*, Suppl., 1938, **95**,

8 Jemerin, E. E., Hollander, F., and Weinstein, V. A., *Gastroenterology*, 1943, **1**, 500.

Vagotomy produced a marked decrease in secretion to histamine stimulation.

In 3 of 4 animals tested, secretion was high in volume and acidity in the fasting state. Within 15 minutes after feeding, there was a pronounced augmentation in secretion, followed in 15 to 30 minutes by a profound inhibition in volume, free acidity, and peptic power. After 3 to 7 hours, secretion returned to the fasting level. Following vagotomy, there was no augmentation with feeding, and the period of inhibition appeared as before.

The ineffectiveness of partial vagotomy was well demonstrated in 2 animals. In one the left, in the other the right vagus nerve was crushed in the cervical region. The secretory response to insulin hypoglycemia was unaffected, and there was no diminution in the 24-hour secretion either in volume or acidity.

Three dogs died of peptic ulcer. Two died of perforation 4 and 7 days after operation; the third died of hemorrhage 5 days after vagotomy.

Conclusions. 1. The totally isolated stomach with intact blood and nerve supply secretes large amounts of gastric juice even in the absence of food-taking.

2. On the ingestion of food there occurs an immediate augmentation of secretion followed by a period of inhibition lasting 3 to 7 hours and then a period of profuse secretion.

3. Section of the vagus nerves above the diaphragm reduces the secretion of gastric juice in the isolated stomach by an average of 56% and the output of hydrochloric acid by 77%. Nervous factors are thus more important than other mechanisms in determining gastric secretion in these animals.

4. Chronic progressive peptic ulcers occur frequently in these isolated stomachs and cause death by hemorrhage or perforation. They rarely develop in such preparations that have been denervated, and following vagotomy, they tend to heal.

5. Partial vagotomy has little or no effect on gastric secretion.

6. After complete vagotomy, the secretory response to a standard dose of histamine is markedly reduced.

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Scarring and Precirrhosis of the Liver in Chronic Phosphorus Poisoning of Guinea Pigs.

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In a previous report,[1] we described the fibrous tissue in dietary cirrhosis of rats and carbon tetrachloride cirrhosis of rats and guinea pigs as forming primarily about the hepatic veins. It was suggested that at least in certain experimental cirrhoses the proliferation and condensation of fibrous tissue occur in the same part of the liver lobule as the parenchymal damage. It seemed desirable to test this concept further by repeatedly injuring the peripheral (portal) part of the liver lobules to determine whether or not fibrous tissue would form about and connect the portal areas.

White phosphorus was selected for use in this study since it has been stated that this substance produces periportal fatty degeneration and necrosis of the liver, and since Mallory,[2] among others, has produced cirrhosis in rabbits with phosphorus.

We were not successful in producing frank liver cirrhosis in the animal used (guinea pig). However, other striking extensive

[1] Ashburn, L. L., Endicott, K. M., Daft, F. S., and Lillie, R. D., *Am. J. Path.*, 1947, **23**, 159.

[2] Mallory, F. B., *Am. J. Path.*, 1933, **9**, 557.

asymmetrical lesions were observed; a description of these changes is the main purpose of this paper.

Experimental Methods. Fifty-one guinea pigs weighing between 300 and 500 g were divided into 2 groups and fed a diet of rabbit pellets and leafy vegetables. The animals in one group received 0.75 mg/kg of phosphorus 4 days each week, while those in the second group were given 1.5 mg/kg twice weekly. The phosphorus was made up as a 0.1% solution in olive oil. This solution was administered orally by the use of a 1 cc tuberculin syringe and a 15-gauge needle one inch long; the end was made slightly bulbous by a small amount of solder. The guinea pigs were held in a vertical position and the proper dose was expelled slowly into the back of the throat. The dose of phosphorus used in the study was, for certain periods, above the tolerance limit of some animals; so, based on the appearance of the animal and the weight curve, it was sometimes necessary to reduce or omit single doses. Occasionally 2 or more successive doses were reduced or omitted to obtain a more satisfactory weight curve. The experiment was continued for a period of 35 weeks. In order to study the anticipated changes as they progressed, from 2 to 4 animals were killed at irregular intervals beginning in the first week.

At autopsy most of the livers were injected as previously described[1] with a charcoal gelatin mass to mark the hepatic or portal veins in the microscopic section. Livers were fixed either in 10% formalin or Helly's fluid, paraffin sectioned, and stained by the Van Gieson technic and with azure eosinate. Some were also stained with hematoxylin and eosin, and by Foot's modification of Rio Hortega's silver method for demonstrating reticulum. In addition frozen sections were stained with oil red O.

Results. An analysis of the results showed no difference in incidence or type of lesions found in the livers of the animals given phosphorus four days a week and those given this substance twice a week; hence all animals will be considered as one group.

Gross Findings. The first gross lesions were seen in an animal killed 9 weeks after the beginning of the experiment. This liver showed, on its ventral aspect, dark reddish brown areas at the hilar portion of most lobes with extension for short distances toward the free borders. The lesions were sharply marginated, depressed, and suggested a loss of parenchymal substance. As the time on the experimental regimen increased, lesions were seen with greater frequency and extensiveness, and involving a greater number of lobes. These lesions varied considerably in size and shape. They were triangular, oval, stellate, or linear, and when isolated they measured up to 2 cm in their greatest dimension. Although they were commonly seen in the hilar region, they were by no means limited to this area. Various lobes of the same or different livers showed variously deep notches along their margins, extreme atrophy of the proximal segment, punched-out areas on dorsal or ventral surfaces, or were incompletely traversed by fissures. When these changes occurred in a single lobe, there was great distortion. Occasionally atrophy and shrinkage of one or more lobes were extreme. When the accessory lobes were involved in this fashion, the gall bladder bed was thus lost and this organ was found in abnormal locations. In one liver the left main and right and left accessory lobes were shrunken to the point where their combined mass measured only 2 x 1 x 0.2 cm (Fig. 1E). The right main and caudate lobes of this liver showed no focal lesions but were considerably enlarged. Hypertrophy was generally present in uninvolved lobes when others showed marked atrophy. The distal third of lobes was rarely involved. This portion was sometimes hypertrophied and connected with the remainder of the liver by a shrunken band of tissue representing the proximal two-thirds of the lobe. Nodular hyperplasia was not seen.

Examination of the cut surface occasionally showed small lesions which did not reach the surface. The externally evident lesions penetrated the liver for varying depths, usually less than half of the thickness of the lobe. At times the entire thickness of the lobe was involved.

The incidence of involvement of the various

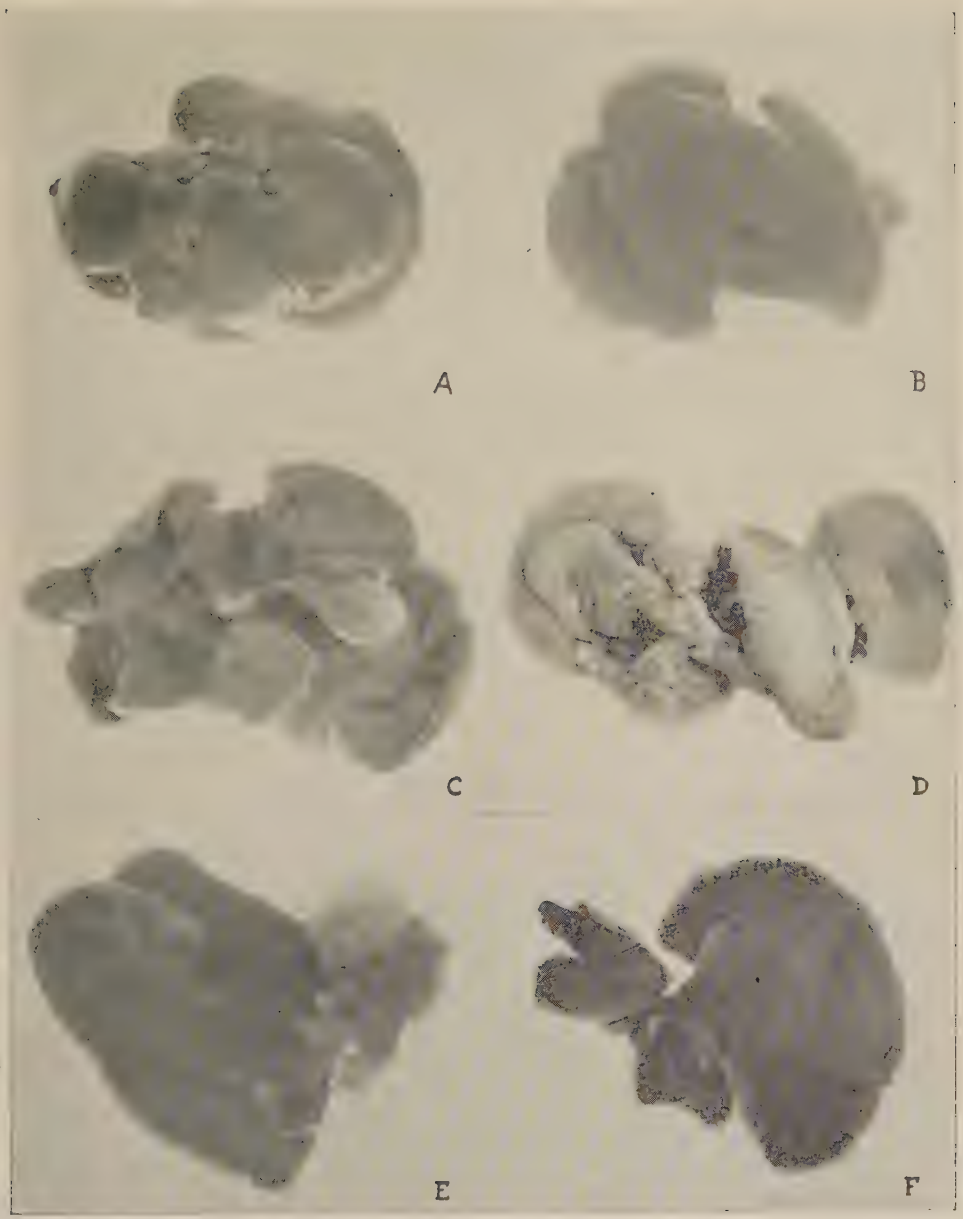

FIG. 1.

Gross photographs of guinea pig livers showing various degrees of deformity following chronic phosphorus poisoning.

A. Diaphragmatic surface—shows defects along margins of lobes.

B. Diaphragmatic surface—similar to A, but more advanced changes.

C. Inferior surface—numerous deep notches and fissures give appearance of nodular hypertrophy. The gall bladder has become detached from its bed (enlarged notch at top) and lies in a horizontal position.

D. Diaphragmatic surface. There is marked atrophy of the right accessory lobe (dark area in center of photograph), fore-shortening of right main, and atrophy of proximal portion of caudate lobe. The atrophic portion of caudate is largely covered by the right main lobe.

E. Inferior surface. The small mass of tissue to the right of, and below the gall bladder is all that remains of the left main and left and right accessory lobe.

F. Diaphragmatic surface. The large mass of tissue is the left main lobe. All other lobes are atrophic.

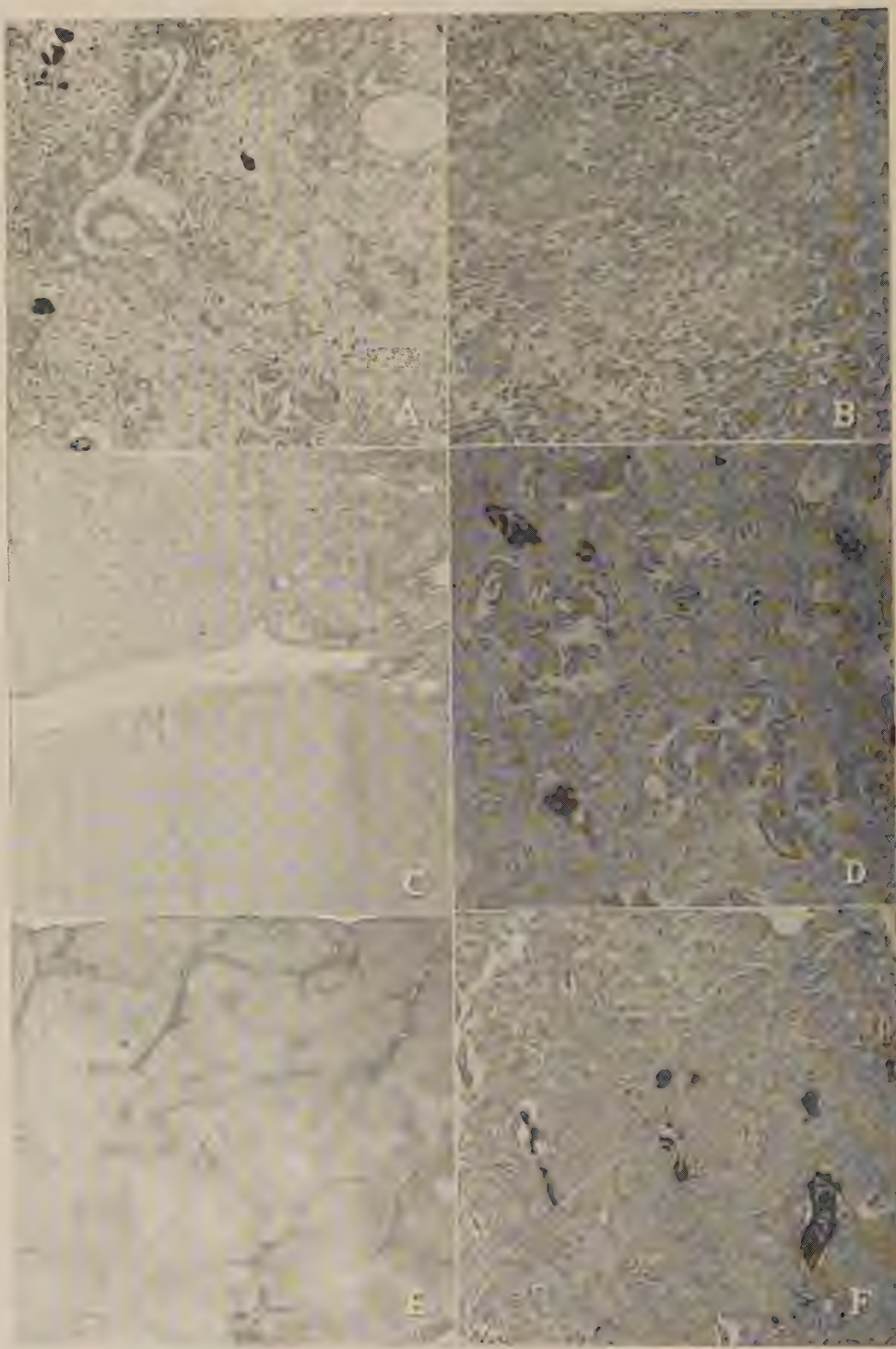

FIG. 2.

Photomicrographs of lesions of guinea pig livers in chronic phosphorus poisoning. Three of the livers were injected with a carbon-gelatin mass.

A. Histologically early lesion showing almost complete disappearance of parenchymal cells,

blood in some sinusoids and no appreciable fibrosis. Hepatic veins were injected. Hematoxylin and eosin stain, × 120.

B. Similar to A but from a different liver. Area was selected to show a few small groups of remaining liver cells and more blood in the non-collapsed sinusoids. Van Gieson stain, × 160.

C. The liver below the hepatic artery is normal. Superficial to it there is subtotal disappearance of liver cells with collapse of sinusoids. Hematoxylin and eosin stain, × 25.

D. Late lesion. Liver cells are replaced by dense fibrous tissue. Note sections of the tortuous hepatic artery. Hepatic veins were injected. Van Gieson stain, × 100.

E. Thin partially fibrous trabeculae connect many portal areas. Prominent hepatic veins are seen within some of the enclosed lobules. Van Gieson stain, × 30.

F. Carbon-gelatin mass was injected in the portal vein. Four portal and three hepatic veins are shown. There is marked reduction in size of lobules but no increase in connective tissue. Azure eosinate stain, × 90.

lobes was irregular, with no particular lobe showing outstanding susceptibility to injury. The right accessory lobe was involved in 22 livers, the left accessory in 19, the right main in 19, the left main in 15, and the caudate in 10. Although the caudate showed the lowest incidence of gross lesions, it revealed the greatest relative incidence of extensive damage. Of the 10 instances of caudate involvement, the lesions were considered extensive in five. This contrasts with the findings in the left main lobe in which only 3 of 15 showed extensive lesions. The other lobes fell between these extremes.

Microscopic Findings. The lesions noted grossly varied in microscopic appearance. This variation was considered as a function of age of the lesions and this interpretation is used to facilitate the description of microscopic appearance. The early lesions were of variable size and showed from moderate to subtotal disappearance of liver cells (Fig. 2, A and B). Sinusoids in most lesions were generally collapsed. In some they were open and blood-filled. In areas of collapse, there was both an apparent and an actual increase in cellularity since large mononuclear cells and occasionally lymphocytes were present in variable numbers. The liver cells remaining in such areas occasionally showed hydropic, fatty or other degenerative changes. Hyperplasia of the remaining cells was not seen. In these early lesions connective tissue fibers were not numerous and reticulum fibers predominated.

In most lesions bile ducts appeared to be present in greater number than could be accounted for by a collapse of lobules. This belief was supported by the presence of biliary epithelium arranged in cords without lumens and by the presence of atypical cords. Bile duct proliferation was never more than moderate and often was only of slight degree.

As the lesions became older they showed an increased amount of fibrous tissue, a decrease in cellularity, somewhat fewer bile ducts and fewer liver cells. What appeared to be the end stage of these lesions was seen in a few livers after the twenty-third week. They showed moderate to marked fibrosis, small to moderate numbers of spindle cells, scattered normal bile ducts and, in some cases, numerous sections of the convoluted or undulating hepatic arteries (Fig. 2D). Occasionally a lesion was seen in which only patchy areas showed fibrosis.

Extensive necrosis was seen in only one animal. This occurred in a liver in which the portal areas were markedly widened due to disappearance of adjacent parenchymal cells, cellular infiltration and proliferation of bile ducts. The majority of the liver cells between such areas were in various stages of karyorrhectic necrosis. Some lobules in this area of involvement showed no liver cells; only blood-filled sinusoids separated the thickened portal areas.

In the vicinity of a few lesions and occasionally elsewhere, liver lobules showed considerable reduction in size without other striking alteration (Fig. 2F). Central veins were approximately centrally located and collagen and reticulum stains showed no appreciable increase in connective tissue fibrils either within the lobules or about central veins or portal areas. There was, however, some disarrangement of the usual radial distribution of cell cords; only rarely was a necrotic liver cell seen in such areas.

In addition to the lesions just described, there were changes which were more widespread. The livers from guinea pigs killed

during the early part of the experiment showed fine droplet fat deposits in the parenchymal cells. In most, it was of moderate degree and distributed diffusely throughout the lobule. In a few, the fat was sharply limited to a zone around the portal areas. This was seen most often when the fat was present in only small amounts. Although fatty change was seen throughout the experimental period (35 weeks), it was occasionally absent and not infrequently was of slight degree, particularly in the last half of the experimental period.

Incomplete hyalinization of liver cell cytoplasm was often present but frank necrosis was limited to a few isolated cells or to very small groups of cells. In a few animals the small number of necrotic liver cells present bordered the portal area but in most livers the necrotic cells were irregularly distributed. In some livers, necrosis was not seen.

Portal cellular infiltrate, composed of lymphocytes and large mononuclear cells, was seen in most animals. When present it usually was of slight to moderate degree and in general was less frequent and less prominent in the latter part of the experimental period.

Proliferation of bile ducts or biliary epithelium was quite irregular, both as to occurrence and prominence. It was evident in less than one-half of the animals and usually was of slight to moderate degree. It was seen most frequently in the first part of the study.

The presence of increased amount of collagen in or about portal areas, although noted earlier in an occasional animal, was seen regularly after the sixteenth week. The collagen deposition was usually slight. In a number of livers after the sixteenth week, the portal areas were broader than normal and projected toward each other between the liver lobules. This tendency was in general progressive and in 4 animals thin trabeculae connected portal areas and surrounded liver lobules (Fig. 2E). Three of these occurred after the thirty-third week of the experiment. This trabeculation did not occur in all lobes and lobule involvement was variable in degree.

The portal changes described did not appear to bear any specific or consistent relationship to the lesions seen grossly. The portal involvement occurred in some lobes showing no gross lesion and gross lesions were present in some lobes in which portal changes were absent or inconspicuous. Occasionally the portal alteration was somewhat more prominent in areas bordering the large lesions.

Discussion. The main purpose of this experiment, the production of clear-cut portal cirrhosis, was not achieved. In 4 of the guinea pigs, certain lobes or parts of lobes showed lobules surrounded by thin trabeculae formed partly of collagen. In a number of other livers, narrow cellular and fibrous portal extensions partly surrounded lobules in an irregular fashion. However, the process was not diffuse, and there was no striking disorganization of lobule architecture and arrangement. Since these latter features were missing, we do not feel justified in claiming the production of portal cirrhosis of the liver by the feeding of phosphorus. Many of these livers were thought of as showing precirrhotic changes. It is possible that frank cirrhosis might have resulted if the experiment had been continued for another six months.

This experiment throws little light on the working hypothesis that repeated zonal damage to the liver parenchyma will be followed by collagen deposition in the involved areas. This is true first because the necrosis was slight, inconstant in occurrence with only a tendency to be portal in location and second because intracellular accumulation of histologically demonstrable fat was often of slight degree and was periportal in location in only about one-half the livers studied.

This study cannot be compared to that of Mallory since in his experiment, in which cirrhosis was produced, rabbits were used almost exclusively. Our choice of the guinea pig as the experimental animal was made in order to avoid difficulties of interpretation which might arise in the rabbit due to the common presence of coccidial fibrosis.

The principal purpose of this paper is to report the occurrence of the focal lesions. We have not previously observed this type of injury following the chronic administration of a hepatotoxic agent.

The early pathogenesis of the focal lesions is not clearly evident. Localized acute necrosis of the parenchyma seems to us the most likely explanation. However, this was seen only in one liver. In evaluating this suggestion it should be borne in mind that cell debris resulting from acute necrosis is often removed from the site in 3 to 4 days. Also it would seem pertinent to recall here that the early necrosis phase of epidemic hepatitis was not observed in the cases reported by Lucké.[3] An alternate explanation of the formation of these localized lesions would be the gradual loss of single or small groups of cells from an area. Against this theory is the fact that generally the entire lesion seemed to be of the same histologic age. Central fibrosis with the peripheral part of the lesion showing only collapsed sinusoids was not observed. It is true that the largest lesions occurred in the latter part of the study but these were considered as probably resulting from the development of adjacent rather than growth of individual lesions. It seems that the very gradual loss of cells from the lobule without replacement would lead simply to a reduction in size of the lobule, without sinusoidal collapse and condensation or proliferative fibrosis; this type of lesion was observed focally in a few livers.

Since histologically "early" lesions were seen throughout the experiment, it cannot be concluded that all lesions would progress to the end stage described above. The "early" lesions seen in the livers of animals killed in the latter part of the experimental period could be chronologically old lesions which had failed to undergo fibrosis. Against this alternative concept is the fact that in general the number of lesions per liver was greater in the second half of the experimental period and that lesions with extensive fibrosis were not seen before the twenty-third week.

A question raised by this experiment concerns the factor responsible for the localized nature of the lesions. It seems quite illogical to assume that the parenchymal cells in one part of the liver are inherently more susceptible to injury by phosphorus than another. It would appear that a more reasonable explanation could be based on some physiologic alteration of blood supply to the area. In an occasional superficial lesion sectioned vertically to the surface, the deep margin of the area of collapse was sharply limited by a fairly large hepatic artery (Fig. 2C). Primary vascular lesions were not observed.

Inasmuch as the hepatotoxic agent was administered by mouth, it is interesting that the gross lesions were neither limited to nor strikingly concentrated in the right or left half of the liver. From this observation, and reasoning from the theory that portal blood from different parts of the gastrointestinal tract reaches different lobes of the liver,[4] it might be suggested that absorption of phosphorus in olive oil is not limited to one segment of the intestines. If the oil phosphorus mixture passed by the lacteals into the general circulation before reaching the liver, this suggestion would be invalid.

It is often difficult to determine whether fibrous tissue, which is seen in some areas where liver cells have disappeared and the sinusoids are collapsed, represents condensation fibrosis or newly proliferated collagen. In the present study we feel that some of the lesions showed amounts of fibrous tissue far beyond that which could be accounted for on a condensation basis. This belief is supported by the fact that the early lesions resembled the microscopic appearance of "acute yellow atrophy" with little or no increase in collagen, even though the sinusoids often were collapsed, whereas the few densely fibrotic lesions were seen only in the latter part of the study. In all likelihood the fibrosis occurred through both mechanisms.

Summary. In an attempt to produce portal cirrhosis, guinea pigs were fed white phosphorus in olive oil by mouth for periods up to 35 weeks. Microscopic examination of livers from animals killed after one week showed inconstant, slight to moderate cytoplasmic hyalinization and occasional to few necrotic liver cells. There was also moderate

[3] Lucké, Balduin, and Mallory, Tracy, *Am. J. Path.*, 1946, **22**, 867.

[4] Bartlett, F. K., Corper, H. J., and Long, E. R., *Am. J. Physiol.*, 1914, **35**, 36.

cellular infiltration of portal areas and parenchymal fatty metamorphosis; the latter was sometimes periportal in location. These changes continued to be observed in most animals throughout the experimental period with increasing amounts of collagen in portal areas appearing as the experiment progressed. The increase in collagen was definite but never great. In 4 animals thin collagenous trabeculae connected portal areas and surrounded lobules. In a number of other animals fibrous extensions of portal areas were present but incomplete. There was neither prominent lobule distortion nor parenchymal hyperplasia. The failure to produce clear-cut portal cirrhosis is considered to be due to the inconstant and minimal degree of periportal necrosis.

After 9 weeks, the livers showed an increasing incidence of depressed gross lesions of irregular shape and size. The lesions in some livers were of such extent and severity as to produce marked deformity and shrinkage of various lobes. Microscopically, the lesions showed moderate to marked loss of parenchymal cells with collapse of sinusoids and reticulum stroma. In the latter part of the experiment a few such lesions showed marked fibrosis. The probable pathogenesis of these lesions is discussed and the incidence by lobes is given.

16304

Hemagglutination by Bacterial Suspensions with Special Reference to *Shigella alkalescens.*

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Agglutination of red blood cells by various viruses has interested many workers, and has been found useful in immunological investigations of virus host relationships. Recently, Keogh, *et al.*[1] have reported agglutination of red blood cells by saline extracts of *Hemophilus pertussis, Hemophilus parapertussis,* and *Hemophilus bronchisepticus.* These authors suggest that this property of *Hemophilus pertussis* is related to the virulence of the organism and its ability to produce protective antibodies in mice.

In the course of an incomplete and unsuccessful survey of bacteria for Rh-like antigens using anti-Rh_0 blood typing serum, it was noted that saline suspensions of certain bacteria agglutinated human red cells in the absence of serum. It is the purpose of this report to describe some aspects of this phenomenon.

Experimental Observations. Hemagglutination by various bacterial species. Saline suspensions of bacteria grown for 24 hours on horsemeat infusion agar (and in a few instances on 5% rabbit blood agar) were prepared to have a density equal to 500 p.p.m. silica standard.[2] This type of suspension was used throughout these studies unless otherwise stated. Equal parts of these suspensions and 2% saline suspensions of once washed human Group O Rh-negative red cells were placed in small tubes. Agglutination was looked for after one hour's incubation at 35°C.

No agglutination of human red cells was observed using suspensions of the following bacteria (wherever more than one strain of organism was examined, the number of strains is given in parenthesis): *Aerobacter aerogenes, Alcaligenes fecalis, Bacillus anthracis, Brucella abortus, B. melitensis, Corynebacterium*

[1] Keogh, E. V., North, E. A., and Warburton, M. F., *Nature,* 1947, **160**, 63.

[2] *Standard Methods for Examination of Water and Sewage,* 6th Edition, American Public Health Association, New York, 1925, p. 4.

diphtheriae, C. hektoenii, Diplococcus pneumoniae (2), *Eberthella typhosa* (3), *Escherichia coli, Klebsiella pneumoniae* (2), *Lactobacillus acidophilus, L. bulgaricus, Pasteurella pestis, P. tularensis, Proteus vulgaris* (3), *P. morganii, Pseudomonas aeruginosa, Salmonella ballerup, S. choleraesuis, S. enteritidis, S. paratyphi* (2), *S. schottmulleri, Serratia marcescens, Shigella ambigua, S. dispar, S. dysenteriae* (2), *S. flexneri* (14), *Staphylococcus aureus* (5), *Streptococcus fecalis, S. pyogenes* (1 of 2 strains), *S. salivarius,* and *Vibrio comma* (3 of 10).

Human red cells were agglutinated by *V. comma* (7 of 10), *S. pyogenes* (1 of 2), *Brucella bronchiseptica, Hemophilus pertussis,* and *Shigella alkalescens* (23).

Clumping of red cells by 7 of the strains of *V. comma,* one strain each of *H. pertussis,* and *S. pyogenes* was detectable only on microscopic examination. The suspension of *B. bronchiseptica* gave clumping clearly visible within 5 minutes by gross examination.

The addition of a saline suspension of *S. alkalescens* to human red cells caused clumping of the cells beginning in about 3 minutes. Microscopically, the cell aggregates resembled those produced by the action of isohemagglutinins. The speed of reaction and the degree of clumping of human cells by *S. alkalescens* was greater than that of other organisms studied. Among the bacteria studied, only *B. bronchiseptica* approached this intensity of agglutination. Further observations of the nature of hemagglutination by *S. alkalescens* are therefore presented.

Effect of Shigella alkalescens on red blood cells of various species. As shown in Table I saline suspensions of *S. alkalescens* N.I.H. No. 4 agglutinate the red cells of certain species of animals and do not agglutinate others.

Agglutination of human red cells by *S. alkalescens* No. 4 does not appear to be related to known factors in the red cells, such as blood agglutinogens A, B, O, M or N, and the Rh antigens, since differences would have been expected to occur among this sample of human cells.

Twenty-one strains* of *S. alkalescens* were tested against cells of man, horses, and hogs. All but one strain gave equally strong reactions with human cells; this weakly reacting strain gave definite microscopic agglutination. Against one specimen of hog cells one strain failed to cause clumping. This strain was a different one from that giving the weak reaction with human cells. All specimens gave negative results with a suspension of horse red blood cells.

TABLE I.
Effect on Red Blood Cell of Several Species of Animals by the Addition of Saline Suspensions of *S. alkalescens* N.I.H. No. 4.*

Species	Type	No. of cell specimens	Result
Human	Group O Rh+	115	+
	" O Rh—	22	+
	" A Rh+	112	+
	" A Rh—	17	+
	" B Rh+	30	+
	" B Rh—	3	+
	" AB Rh+	3	+
	" AB Rh—	2	+
Monkey	(*M. rhesus*)	17	+
Hog		8	+
Horse		5	—
Dog		8	—
Cow		15	—
Sheep		2	—
Rabbit	New Zealand	37	—
Guinea pig		25	—
Mice	Swiss	5	—
Hamster		5	—
Rat	Wistar	5	—

* Mixtures of equal parts of 2% red cell suspensions and bacterial suspensions having turbidity equal to 500 p.p.m. silica standard, observed 1 hour at 35°C.

Rh designations refer to the results obtained using an Anti-Rh_o blood typing serum.

— = no agglutination.

\+ = macroscopic agglutination.

Effect of various factors on the hemagglutinin of Shigella alkalescens. Broth cultures of *S. alkalescens* N.I.H. No. 4 were filtered through a Berkefeld filter, and the clear filtrate gave no reaction with human red cells. The possibility that the hemagglutinin was intimately associated with the bacterial cell was investigated.

A heavy suspension of *S. alkalescens* N.I.H. No. 4 in saline was exposed to sonic vibration at 8,000 oscillations per second for 20 min-

* Obtained from the Bacteriology Department of the U. S. Naval Medical Center, Bethesda, Md.

TABLE II.
Effect of Heat, Phenol, and Formalin on the Ability of *S. alkalescens* N.I.H. No. 4 to Agglutinate Human Red Cells.*

Treatment of suspension of *S. alkalescens* No. 4	Agglutination of human red cells Dilution of bacterial suspension						
	undil.	1:2	1:4	1:8	1:16	1:32	1:64
Fresh suspension	+++	++	++	+	+	±	—
37°C for 2 hr	+++	++	++	+	±	—	—
56 " " 5 min	+++	++	++	+	+	±	—
56 " " 15 min	+++	++	+	+	—	—	—
56 " " 30 "	++	+	+	—	—	—	—
93 " " 1 "	—	—	—	—	—	—	—
0.5% formalin 30 min	+++	++	++	+	±	—	—
0.5% phenol 30 min	+++	++	++	+	±	—	—

* Bacterial suspensions equal in turbidity to 500 p.p.m. silica standard. 2% saline suspensions of red cells.
— = no agglutination.
± = microscopic agglutination.
+ = macroscopic agglutination

utes. Supernatant fluid from this suspension when undilute gave only microscopic reactions. Similar heavy suspensions of the organism were then rapidly frozen (in dry ice cellusolve mixture) and thawed 12 times, after which the material was placed in a Sharples centrifuge and spun at approximately 18,000 r.p.m. The supernatant resulting from this treatment gave reactions with human cells when diluted 256 times.

It was noted that human red cells could be washed 5 times with saline without affecting the degree of reaction with *S. alkalescens*. However, suspensions of *S. alkalescens* N.I.H. No. 4 washed with saline showed a progressive loss of hemagglutinin for human cells. Traces of hemagglutinin present after 3 washings disappeared after 6 washings.

These observations indicated that the hemagglutinin was separable from the bacterial cell, and therefore, the factor may have been adsorbed to the filter as previously used. Efforts to wash the material from Berkefeld filters were unsuccessful.

The effect of heat, phenol, and formalin on the agglutination factor of *S. alkalescens* is summarized in Table II.

Heat had an adverse effect on the hemagglutinin, causing slight loss of activity at 37°C for 2 hours, more noticeable effects at 56°C for 15 minutes, and destroying activity in one minute at 93°C. The addition of phenol and formalin to bacterial suspensions appeared to lessen the activity of the hemagglutinin after 30 minutes contact.

Agglutination of human red cells by *S. alkalescens* took place at 5°C and at 56°C as readily as at room temperature.

The production of hemagglutinin by cultures of *S. alkalescens* at 35°C is shown in Table III. Bacterial suspensions of comparable turbidity prepared on various days of growth on agar medium showed that hemagglutinin increased from its level at 2 days to maximum proportions in 5 to 7 days, and thereafter fell in titer.

The possibility that agglutinins for human cells present in various animal serums and tissue juices may have influenced the reactions observed, appeared less probable when *S. alkalescens* grown on a medium containing a pancreatic digest of casein gave equally good reactions.

Antiserums, prepared by injecting formalinized suspensions of *S. alkalescens* into rabbits, agglutinated the organisms. The agglutinated organisms washed free of serum failed to agglutinate human cells.

A phenomenon not well explained was observed in the course of this study. Twenty-three strains of *S. alkalescens* on horsemeat infusion agar were placed in a cold room (5°C) for approximately 6 weeks. At this time, transplants were prepared and from these actively growing organisms suspensions were made, none of which agglutinated human

TABLE III.
Titration of Hemagglutinin in Suspensions (Turbidity = 500 p.p.m.) of *S. alkalescens* on Different Days of Growth*

	Agglutination of human red cells Dilution of bacterial suspension							
Day of growth	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
2nd	++	++	+	—	—	—	—	—
3	+++	++	++	+	—	—	—	—
5	+++	+++	++	++	++	+	±	—
6	+++	+++	++	++	++	+	±	—
7	+++	++	++	+	+	+	+	—
8	+++	++	++	+	+	±	—	—
9	+++	++	+	±				

* Bacterial suspension prepared each day from growth of *S. alkalescens*. Turbidity = 500 p.p.m. silica standard. 2% saline suspensions of red cells.
— = no agglutination.
± = microscopic agglutination.
+ = macroscopic agglutination.

red cells. Additional dried cultures (such as were used originally in these studies) were obtained and fresh transplants from the dried cultures showed agglutinating activity as noted before. Thus it would appear that the hemagglutination factor is labile and under certain conditions may disappear from strains known to have possessed this factor.

Discussion. The phenomenon of hemagglutination by bacteria as reported here appears to be different from reactions reported by Huebner, Thomsen, Friedenreich,[3] and Terada.[4] These authors observed that red cell suspensions in which certain strains of bacteria (*Corynebacterium* and gram-positive cocci) are allowed to grow become agglutinable with all human serums used to test the red cells. Nor does the phenomenon reported here appear to be related to the action of *Corynebacterium hektoenii*,[5] or a mustard bacillus,[6] which impart to certain serums in which they are grown the ability to agglutinate all human and some animal red cells.

The ability of bacterial suspensions to agglutinate red blood cells reported by Kraus and Ludwig[7] in 1902 and later elaborated by Guyot[8] and Fukuhara[9] appears to have received little attention.[10] Kraus and Ludwig reported that certain strains of staphylococci and vibrio agglutinated rabbit cells. They reported that hemagglutination by organisms could be interfered with by the action of specific antiserum. Guyot reported that 12 of 18 strains of organisms designated *B. coli* agglutinated the cells of various animals. Eight of these strains showed considerable difference in the species of animals with which they reacted. He was unable to separate hemagglutinin from the bacterial cell. Fukuhara found that diphtheria, mouse typhoid, paratyphoid, and Friedlander's bacilli and Sarcina contained hemagglutinins for cells of some species of animals. He extracted the hemagglutinin with alcohol from bacterial suspensions treated with 0.1N NaOH. The possibility that such reactions might be used in immunological studies of bacteria, as suggested by Keogh *et al.*, gives bacterial hemagglutinin added significance.

The finding of an active hemagglutinin in cultures of *S. alkalescens* provides a readily grown source of hemagglutinin for the study of the phenomenon of agglutination. In ad-

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[4] Terada, K., *Taiwan Iyakkai Zasshi*, 1936, **35**, 1267.

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[6] Grove, E. F., and Crum, M. J., *J. Lab. and Clin. Med.*, 1930, **16**, 259.

[7] Kraus, R., and Ludwig, S., *Wien. Klin. Wchnschr.*, 1902, **15**, 120.

[8] Guyot, G., *Cbl. f. Bakteriol.*, 1908, **47**, 640.

[9] Fukuhara, Y., *Z. f. Immunitätsforsch.*, 1909, **2**, 313.

[10] Pearce, R. M., and Winne, C. K., *Am. J. Med. Sci.*, 1904, **128**, 669.

dition, the presence of hemagglutinin may be useful in distinguishing this bacterial species from others of the genus *Shigella*.

The specificity of the action of *S. alkalescens* on human, monkey, and hog red blood cells appears to indicate that some substances common to these three species are present in their red cells, while absent in cells of other animal species. This specificity is in rather marked contrast to the findings of Guyot, and also to the recent report of Keogh.

Summary. 1. Saline suspensions of *S. alkalescens, B. bronchiseptica, H. pertussis, V. comma, S. pyogenes* are capable of clumping human red blood cells.

2. Suspensions of *S. alkalescens* agglutinate human, monkey, and hog red blood cells, and do not agglutinate cells of certain other animal species.

3. The hemagglutinin of *S. alkalescens* can be separated from the bacterial cell by high speed centrifugation after successive freezing and thawing of bacterial suspensions.

4. The hemagglutinin of *S. alkalescens* is labile, being destroyed by heat, and spontaneously disappears from cultures on standing at 5°C for 6 weeks.

16305

Effect of Thyroxin on Mouse Susceptibility to Polioencephalitis.*

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The fact that a small fraction of persons in a population group display recognizable symptoms of poliomyelitis even in epidemic situations has led to the suggestion that there may be physiological factors involved in determining the susceptibility to the disease.

The higher morbidity rate among children and pregnant women and the possibility of predisposing factors such as physical exertion and preceding infections suggest that this hypothetical physiological factor may be of an unspecific character. An increased metabolic rate is common to all these conditions mentioned. However, it may be noted that the observation of general decreases in BMR during the warmer season of the year when most poliomyelitis epidemics occur is not in agreement with the assumption of an increased metabolic rate as a factor increasing the susceptibility to poliomyelitis.

Recent experimental studies[1] on the influence of environmental temperature on the resistance of Swiss white mice to the poliomyelitis virus showed a marked tolerance of these animals to the infection when acclimated to low temperatures. Since thyroxin secretion is increased upon exposure to cold the influence of thyroid treatment on the susceptibility to poliomyelitis was investigated. On a small number of Swiss white mice it could be shown[2] that thyroactive substances were able to prolong the incubation period of poliomyelitis up to 100% whereas thiouracil had the opposite effect.

The effect of the thyroid hormone on the resistance to neurotropic virus disease is of great theoretical and practical importance and therefore further studies on this subject seemed indicated. Our investigations were concerned with the effect of crystalline thyroxin on the susceptibility of mice to the MM strain of mouse polioencephalitis. Thyroxin was chosen instead of other thyroactive extracts or substances because the effect of crystalline thyroxin on the metabolism lends itself in a more accurate way to a standardization of experimental conditions.

The 3-5-weeks-old mice used for these

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Holtman, D. Frank, *Science,* 1946, **103**, 137.

2 Holtman, D. Frank, *Science,* 1946, **104**, 50.

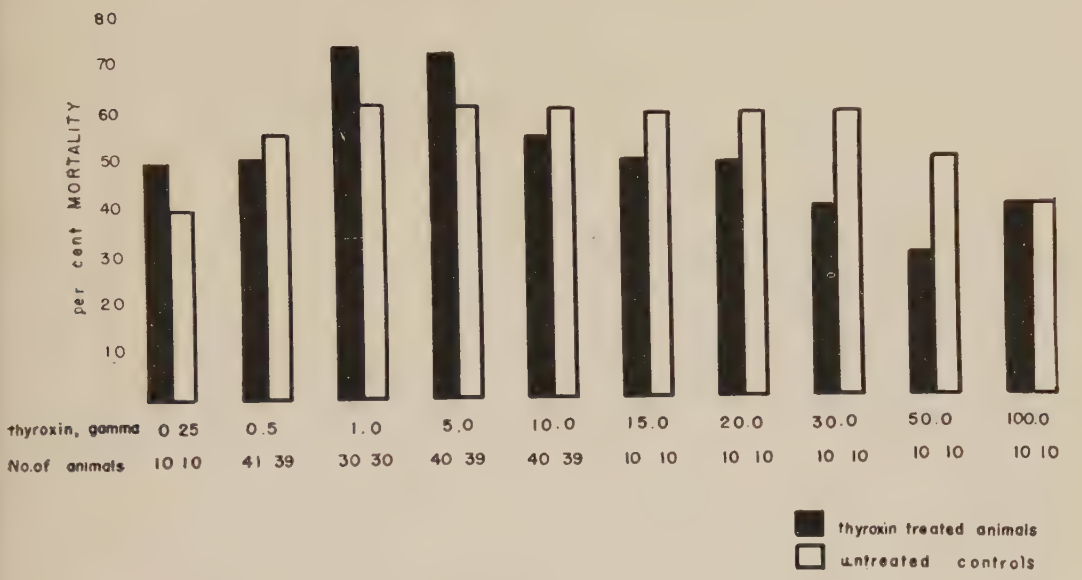

FIG. 1.
Effect of Various Doses of Thyroxin on Mouse Polioencephalitis.

experiments were of the AB strain and came from the colony of Dr. John Bittner, University of Minnesota. Their food consisted of Purina fox chow. The experiments were carried out during the months of March, June, and July.

Fifty mg of thyroxin crystals containing not less than 64% iodine were dissolved in 5 drops of N sodium hydroxide and diluted with distilled water to 50 cc. Injections of thyroxin were made subcutaneously four days before the inoculation with the virus so that the height of the thyroxin effect would coincide with the end of the incubation period.

The MM mouse virus was obtained from Dr. Raymond Bieter, University of Minnesota. 0.1 cc of a 10^{-6} dilution of the virus preparation was injected intraperitoneally without anesthesia. The same dilution was used throughout the experiments.

All animals which showed signs of paralysis and encephalitis or were found dead within the period of 24 hours to 2 weeks after the injection of the virus were considered to have succumbed to the disease.

The results in Fig. 1 show that no dose of thyroxin has been found which could protect the animals to a significant degree. Whenever differences in the susceptibility to the disease between the thyroxin-treated and untreated control animals seemed to occur, these differences were found insignificant when a larger number of animals were used.

TABLE I.
Mortality of Thyroxin-treated Animals and Untreated Controls.

	Thyroxin-treated animals	Non-treated controls
% mortality after one week	35.0	39.7
% mortality after 2 weeks	51.2	55.6
No. of animals	211	207

Table I summarizes the result of all experiments on thyroxin-treated and untreated control animals. No change in the length of the incubation period and no significant difference in the mortality between thyroxin-treated and untreated animals could be observed.

Summary. The susceptibility of young mice of the AB strain to polioencephalitis due to the MM virus could not be affected significantly by treatment with various doses of crystalline thyroxin.

16306

Purification of the MM Poliomyelitis Virus.*

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In the numerous attempts to purify different strains of the poliomyelitis virus many methods have been employed including adsorption,[1] ultracentrifugation,[2,3,4] salting out and ultracentrifugation,[5,6,7] isoelectric precipitation, salting out, and ultracentrifugation,[8] isoelectric precipitation and ultracentrifugation,[9] freezing and thawing and ultracentrifugation,[10] and precipitation with acetone.[11]

In recent years the use of organic solvents as precipitating agents has found wide application in the purification of various proteins. It has been shown that methyl alcohol at low temperatures loses its denaturing effect on proteins[12] and that conditions of alcohol concentration, ionic strength, temperature, and protein concentration have to be controlled in order to achieve optimal separation.[13] For the purification of tetanus toxoid[14] and diphtheria toxoid[15] these conditions have been established. The successful purification of different strains of the influenza virus by alcohol precipitation at low temperatures has also been reported[16] and the same authors observed that essentially the same procedure may be used for the purification of other viral and rickettsial agents, regardless of whether the starting material was infected allantoic fluid, yolk sac, chick embryo, mouse or rabbit brain.

The present work deals with the purification of the rodent polioencephalomyelitis virus known as the MM strain,[17] by methods which use freezing and thawing, alcohol precipitation and dialysis as their main features. After numerous preliminary experiments the following method gave the purest preparation with the best yield.

A 33% mixture of infected mouse brain and cord in 0.1 M phosphate buffer pH 7.0 is prepared by homogenizing the previously frozen tissue in a Potter-Elvehjem glass homogenizer. The mixture is then frozen at −20°C for 24 hours. After thawing the tissue particles are centrifuged off at 4,800 RPM at 4°C for one hour. The clear supernatant fluid is shaken with an equal volume of ether and centrifuged at 4,800 RPM in the cold for 15 minutes. The gelatinous layer at the surface is pierced with a glass rod, the clear fluid underneath is collected in a beaker, the remaining ether is suctioned off in a desiccator connected with a water pump for 30 minutes and the clear solution is then transferred into centrifuge tubes and frozen at −20°C over night. After thawing to 0°C, the insoluble material is centrifuged off at 4,800 RPM in the cold for one hour and discarded.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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TABLE I.
Nitrogen Content, Virus Activity, and Virus Yield During Purification of MM Poliomyelitis Virus.

	G nitrogen/ml	LD_{50}/ml	G nitrogen/LD_{50}	Yield in %
Original solution	.00347	107.9	$3.4 \times 10^{-10.9}$	100
Fraction 1	.00270	107.8	$2.7 \times 10^{-10.8}$	82
" 2	.00063	109.2	$6.3 \times 10^{-13.2}$	74
" 3	.000397	109.0	$3.9 \times 10^{-13.0}$	66
" 4	.000207	109.2	$2.0 \times 10^{-13.2}$	67
" 5	.00014	109.1	$2.4 \times 10^{-13.1}$	65
" 6	.00008	108.8	$8.0 \times 10^{-13.8}$	65

The clear supernate (fraction 1) is diluted 15 times with isotonic saline solution, the pH is then adjusted with N/10 hydrochloric acid by means of glass electrodes to 5.9 and the temperature of the solution is lowered to –1°C by immersing the beaker in a constant temperature cold bath. Enough methyl alcohol chilled to –20°C and measured at that temperature is slowly added through a capillary to make up to 32% of the solution. The alcohol is left in contact with the solution for at least 6 hours and the precipitate is centrifuged off at 4,800 RPM in the cold for one hour. The clear supernatant fluid is discarded, the precipitate drained in the cold and washed once with a chilled mixture of 32% methyl alcohol in isotonic saline solution of pH 5.9. The precipitate is again drained in the cold and then thoroughly suspended in one-twentieth of the original volume of phosphate buffer at room temperature and stirred with an electric stirrer for one hour. The insoluble particles are centrifuged off at 4,800 RPM in the cold for one hour and discarded. The supernate (fraction 2) is frozen at –20°C for 24 hours. After thawing, but still at 0°C, the formed precipitate is centrifuged off at 4,800 RPM in the cold for 30 minutes and discarded. The clear opalescent supernate (fraction 3) is diluted 10 times with isotonic saline solution, the pH is adjusted to 5.5 with N/10 hydrochloric acid, the temperature is lowered to –1°C and enough chilled methyl alcohol is added to give a final concentration of 30%. After at least 6 hours, the precipitate is centrifuged off in the cold for 30 minutes and the supernate is discarded. The precipitate is drained in the cold and washed with a chilled solution of 30% methyl alcohol in isotonic saline pH 5.5, again drained in the cold and then thoroughly suspended in one-tenth of the original volume of phosphate buffer pH 7.0 at room temperature and stirred with an electric stirrer for one hour. The insoluble particles are centrifuged off for one hour in the cold at 4,800 RPM and discarded. The supernatant fluid (fraction 4) is frozen over night. After thawing, but still at 0°C, the precipitate formed is centrifuged off in the cold at 4,800 RPM for one hour and is discarded. The water-clear supernatant fluid (fraction 5) is transferred into cellophane tubing of 8 mm diameter and dialyzed against frequent changes of distilled water in the cold for one day. After centrifuging the solution in the cold at 4,800 RPM for one hour a small precipitate of water insoluble proteins can be separated and discarded. The water clear supernatant fluid (fraction 6) contains the purified virus.

The nitrogen content of all fractions was measured by the micro Kjeldahl method. The activity of each fraction was ascertained by intracerebral injection of 0.03 ml of the diluted fractions into 3- to 5-weeks-old mice of the ZBC strain obtained from the colony of Dr. John Bittner, University of Minnesota. The animals were observed for 2 weeks. Groups of 8 mice were used for each dilution and the final LD_{50} was calculated by the method of Reed and Muench.[18]

The results of a typical experiment and the effect of each step on the nitrogen content and the specific virus activity are shown in

[18] Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

Table I. In this experiment a 43-fold purification was achieved and after the deduction of the 35% loss of virus activity a purification factor of about 28 resulted.

Conclusion. A simple method requiring standard laboratory equipment only is described which by means of freezing and thawing, alcohol precipitation and dialysis accomplishes a marked purification of the MM poliomyelitis virus.

16307

Electronmicroscopy of the Purified MM Poliomyelitis Virus.*

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With the technical assistance of Miss Virginia Mary Kletzin.
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The purified virus used for electronmicroscopic studies was prepared in essentially the same way as described in detail in the preceding paper. During the preliminary studies the observation was made that highly concentrated brain suspensions in phosphate buffer yielded heavier precipitates after freezing and that a higher degree of purification could be achieved by keeping the protein concentration as high as possible.

A 25% mixture of infected mouse brain and cord in 0.1 M phosphate buffer pH 7.0 was prepared by homogenizing the previously frozen tissue in a Potter-Elvehjem glass homogenizer. The mixture was then frozen for 24 hours. After thawing the tissue particles were centrifuged off, the clear supernate was extracted with two-thirds its volume of ether, centrifuged and the gelatinous layer at the surface was discarded. The remaining ether was removed in a desiccator and the solution was frozen overnight. After thawing the precipitate was centrifuged off and discarded. The temperature was then lowered to $-1°C$ and enough chilled methyl alcohol was added to a final concentration of 30% methanol. After 6 hours the precipitate was centrifuged off, washed once with 30% methyl alcohol in buffer, suspended in one-tenth of the original volume, the insoluble particles were centrifuged off and the supernate was frozen overnight. After thawing the precipitate was centrifuged off and discarded. The supernatant fluid was dialysed against distilled water for 24 hours, the water insoluble proteins were centrifuged off and discarded. The supernatant fluid was frozen for 2 weeks, thawed and a final precipitate was centrifuged off and discarded.

By this procedure the nitrogen content was reduced from 2.72 mg per ml in the original clear solution to 0.0119 mg per ml in the final fraction. Thus, a 228 fold purification was achieved or 99.5% of the total nitrogen of the original solution was removed. The final yield amounted to about 10% of the total virus activity of the original solution and therefore a purification factor of about 22.8 resulted. The final fraction contained 1.19×10^{-12} g of "virus" nitrogen per ml and one inoculum contained 3.9×10^{-14} g of "virus" nitrogen.

The mounts for the electron micrographs were prepared by the usual technique of applying a small drop of the 10 times diluted final fraction (1.19×10^{-13} g nitrogen per ml) to a thin formvar membrane supported by a 1/8 inch diameter disc of 200 mesh copper screen and allowing it to dry. The gold-shadowed specimen was prepared by applying a small drop of the solution to a microscope slide. This was treated by the gold shadowing technique of Williams and Wyckoff[1] (8 AU film of gold at a shadow angle of 5 to 1) stripped with collodion and mounted on the copper screen. Measurement of the par-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

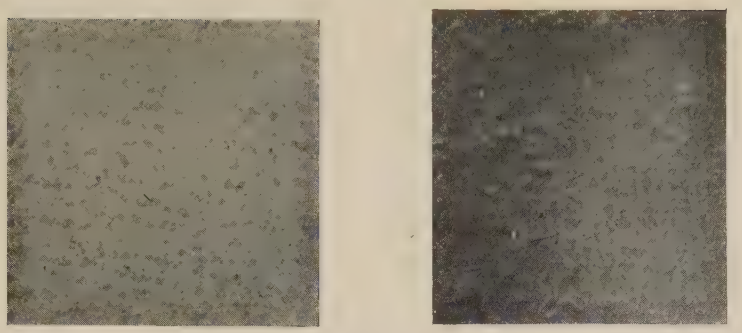

FIG. 1.
Electron micrographs and shadowgraphs of an infected mousebrain suspension containing 3.9×10^{-14} g nitrogen per LD_{50}. Magnification 30,000.

ticle dimensions could be made by measuring the shadow width and shadow length. The shadow length should be 5 times the diameter.

In both micrographs there is an indication for a slight asymmetry of the particles. In measuring the diameter of the particles a minimum dimension of 10 mμ and a maximum of 20 mμ with an average of about 12 mμ was found.†

These micrographs confirm the findings of Loring, Marton and Schwerdt[2] of the absence of thread-like particles[3] and show also essentially the same shape and size of particles as described by these authors.

Summary. Electronmicroscopic studies of a preparation of purified MM Poliomyelitis virus show an uniformity of particle size in the range from 10 to 20 mμ with an average diameter of about 12 mμ.

† These measurements would indicate a molecular weight of the order of 500,000 to 1,000,000.

2 Loring, H. S., Marton, L., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 291.

3 Gard, S., *Acta Med. Scand.*, Suppl., 1943, **143**, 173.

16308

Serum and Plasma Antithrombin.*

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For years it has been known that blood has the ability to inactivate added, as well as spontaneously formed, thrombin. Rettger[1] attributed this to the plasma proteins as a specific function; Lenggenhager[2] related the activity to the albumin fraction. Smith's[3] group has suggested that the *rate* of thrombin destruction varies with the heparin concentration and that the absolute *amount* is limited by the concentration of heparin "cofactor." Wilson[4] developed a method for estimating antithrombin by so diluting plasma

* Abridgment of a portion of thesis, submitted by Dr. Owen to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D. in Medicine.

1 Rettger, L. J., *Am. J. Physiol.*, 1909, **24**, 406.

2 Lenggenhager, Karl, *Helv. med. Acta*, 1935, **1**, 527.

3 Seegers, W. H., Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Science*, 1942, **96**, 300.

4 Wilson, S. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 676.

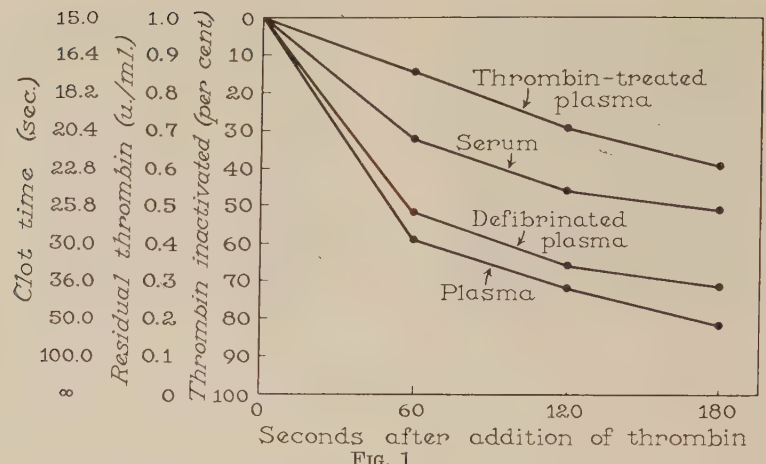

FIG. 1.
Deterioration of thrombin added to diluted serum or plasma.

that it inactivates 1 unit of thrombin in 4 minutes. Astrup and Darling,[5] adding an excess of thrombin to a small amount of plasma, await maximal inactivation before measuring the residue. By both methods no difference in the antithrombic activity of serum and plasma was detected, in spite of the fact that serum inactivates about 300 thrombin units per milliliter in its development.

In an attempt to increase the sensitivity of measurement of thrombin inactivation, the following method was devised. Serum or oxalated plasma is diluted tenfold with buffered saline solution (1 part isosmotic imidazole buffer,[6] 9 parts 0.9% sodium chloride). To 1.0 ml of this solution is added 0.1 ml of thrombin (11 units per milliliter), to make a final thrombin concentration of 1 unit per milliliter. In the case of plasma, fibrin begins to form in 20 to 30 seconds; sharp agitation of the tube clumps the fibrin and prevents its obscuring the final step. Exactly 60 seconds after the addition of thrombin, and in some cases at intervals thereafter, 0.4 ml of the solution is added to 0.1 ml of 0.5% buffered fibrinogen solution, and the coagulation time is determined. The amount of residual thrombin is calculated by comparison of the clot times with the times found for dilutions of thrombin with buffered fibrinogen solutions (Fig. 1).

Tests were performed at 24° to 26°C. Parke-Davis beef thrombin and Armour's Fraction I (fibrinogen) were routinely employed, but were checked against homologous preparations, with negligible variation in the results.

By the use of this method it was found that oxalated plasma (diluted 1:10) from 12 normal dogs inactivated about 60% (40 to 82) of the added thrombin in 60 seconds (Fig. 1). Serum (diluted 1:10) from the same blood samples inactivated about 25% (14 to 38) in 60 seconds. Oxalation of the serum, or preliminary defibrination of the plasma by the addition of only a fraction of a unit of thrombin, altered the results only slightly. The significance of the amount of thrombin deterioration may be estimated from this experiment: a 1:2 dilution of pooled normal dog serum inactivated 81% of added thrombin in 1 minute; 1:3 serum, 67%; 1:4 serum, 50%; 1:5 serum, 39%; 1:6.7 serum, 28%; 1:10 serum, 23%; 1:20 serum, 10%; 1:40 serum, 7%; and 1:100 serum, trace.

To examine the serum-plasma difference further, dicumarol was administered intravenously to dogs; blood samples were collected periodically. Along with each sample preserved by oxalate, another specimen was

[5] Astrup, Tage, and Darling, Sven, *Acta physiol. Scandinav.*, 1942, **4**, 293.

[6] Mertz, E. T., and Owen, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 204.

[7] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

TABLE I.
Antithrombic Activity of Plasmas and Serums from Two Dicumarol-treated Dogs (A and B).

% prothrombin in plasma*		% thrombin deterioration in 60 seconds	
A	B	Plasma	Serum
100 (320 units per ml)		59	32
	100	61	36
	77	64	43
71		60	35
	66	67	38
52		68	45
	45	60	54
43		63	53
	33	66	58
31		68	56
	24	62	58

* Two-stage titration.[7]

TABLE II.
Antithrombic Activity of Plasma at Various Stages During Spontaneous Clotting.

Time after collection of blood, min	% prothrombin*	% thrombin deterioration in 60 sec.
Immediately	100 (305 units per ml)	61
3	68	53
5	52	48
7†	36	44
9†	21	40
11†	3	33
15†	1	33

* Two-stage titration.[7]
† Fibrinogen-free.

set aside for development of serum. Table I shows the antithrombic activity of comparable plasmas and serums from 2 dogs. If antithrombic determinations had been made on serums alone, the evidence would suggest more antithrombin in serums from the low prothrombin samples than in serums from the high prothrombin samples. From study of the oxalated plasmas alone, no difference was seen.

Somewhat similar results are noted in the following experiment (Table II). Fifty milliliters of normal dog blood were collected; at intervals 4.5 ml were added to 0.5 ml of 1.85% potassium oxalate. Clotting began at about the sixth minute; thus the last 4 specimens were fibrinogen-free. It would seem that serum has an antithrombic activity varying inversely with the amount of thrombin to which it has been exposed during clotting.

If this is correct, addition of sufficient thrombin to plasma should simulate the effect of spontaneous clotting. One milliliter of oxalated plasma was mixed with 1.0 ml of 500 units per milliliter thrombin. In 2 hours no thrombin was demonstrable. After appropriate dilution the antithrombic activity of this solution was less than that of control serum: thrombin-treated plasma, 14% inactivation in 60 seconds; serum, 30% (Fig. 1).

Summary. A method of estimating the rate of natural antithrombic activity is suggested. When this method was used, dog plasma, regardless of the prothrombin level after dicumarolization, seemed to exert about the same antithrombic activity. On the other hand, dog serum antithrombin tended to vary inversely with the prothrombin content of its original plasma, and serum obtained from blood containing little prothrombin was antithrombic to about the same extent as plasma. These observations suggest that antithrombic activity be determined on plasma unless due consideration is given to the antithrombic activity already performed by serum.

16309

Kidney Alkaline Phosphatase of Rats Following Alloxan Induced Diabetes and Acute Hypo- and Hyperglycemia.

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It has been assumed that kidney phosphatase hydrolyzes a phosphate ester of glucose during the reabsorption of sugar from the glomerular filtrate. The following experiments were undertaken to determine whether the quantity of alkaline phosphatase of the kidney cortex, located principally in the proximal convoluted tubules, could be altered by varying the amount of glucose available in the tubules for reabsorption. Alterations in the glucose concentration of the body fluids, and presumably of the glomerular filtrates, were achieved in 3 ways.

One group of rats was made diabetic with alloxan and studied at intervals up to 6 weeks after administration of the drug; another group was made hypoglycemic with insulin; and a third group was made hyperglycemic by glucose administration.

The alkaline phosphatase activity was determined on an extract of renal cortical tissue, and the kidneys were examined for alkaline phosphatase content histochemically. A control series of rats was similarly studied.

Experimental procedure and methods. Rats of 4 different strains (Long-Evans, Hisaw, Wistar, and mixed) and of both sexes were used. No variation attributable to these factors was found. Blood sugar analyses by the micromethod of Folin[1] were made on many of the animals at the time they were sacrificed. In addition, determinations of reducing substances in the urine were carried out on most of the diabetic rats and on those given large amounts of glucose.

Seven rats constituted the control series. The experimental animals comprised 3 groups: *Group 1—Rats with alloxan diabetes.* Seven rats were given one dose of 200 mg/kg of alloxan intraperitoneally. Subsequently, some were maintained on 2 to 3 units of insulin daily up to 3 to 4 days before the conclusion of the experiments. The others were given no insulin. *Group 2—Hypoglycemic rats.* Two rats were given 6 units per kilo of regular insulin and 5 units per kilo of protamine insulin. The doses were repeated after 3½ hours. The animals went into collapse 4 hours after the first dose and frequent hypoglycemic convulsions followed. The experiment was terminated 2 to 3 hours later. *Group 3—Hyperglycemic rats.* Four rats were given 7.5 g of glucose over a 12-hour period by subcutaneous injections at 2-hour intervals of a 10% solution of glucose in modified Ringer's solution (composition: 100 ml of water, 10 g glucose, KCl 0.5 mM, $CaCl_2$ 0.1 mM, $NaHCO_3$ 2.5 mM, NaCl 8.0 mM).

Method for determining alkaline phosphatase activity. The activity of the alkaline phosphatase of the kidney cortex was determined on an extract of the dried tissue, and the results were expressed as milligrams of phosphorus hydrolyzed per hour per g of dried kidney weight. β-glycero-phosphate was used as the substrate, without magnesium according to Bodansky's method,[2] and also with magnesium added as activator.

Preparation of the extract. The method of Fischer[3] was adopted with some modification as appears below. Fresh renal cortex was snipped into small pieces and dried overnight in a vacuum desiccator over NaOH. The tissue was then pulverized in a mortar and an aliquot dried in an oven at 110°C

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† Present address: Department of Pathology, Yale Medical School, New Haven, Conn.

1 Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1930, **88**, 85.

2 Bodansky, A., *J. Biol. Chem.*, 1933, **101**, 93.

3 Fischer, Clary J., unpublished data.

TABLE I.
Kidney Alkaline Phosphatase Determinations in Normal Rats and in Alloxan Diabetes. Results Expressed as mg Phosphorus Released per Gram Tissue Solids per Hour.

Normals		Alloxan diabetics			
Rat No.*	mg P†	Rat No.*	Time after alloxan, days	Blood sugar, mg %	mg P†
1a	26	8a	7	—	2.0
2	25	8b	7	—	1.7
3	17	9a	11	—	5.1
4a	62	10	12	500	1.9
4b	66	11‡	21	—	27-48
5a	33-45	12‡	21	602	10-14
5b	47-66	13‡	21	612	12-21
6	22-34	14a	42	764	12-17
7	27-45	14b	42	764	13-17

* Nos. with *a* or *b* indicate assays of individual kidneys. Other numbers refer to pooled kidney analyses.

† Where 2 figures are given, the first is the result obtained without added Mg; the second with added Mg. Where only one figure is given, no Mg was added.

‡ Animals 11, 12, and 13 received no insulin. Others of this alloxan-treated group received 2-3 units per day until 3-4 days before animals were sacrificed for analysis.

TABLE II.
Rat Kidney Alkaline Phosphatase Determinations in Experimental Hypo- and Hyperglycemia Expressed as mg P Released per g Tissue Solids per Hour.

Hypoglycemics			Hyperglycemics		
Rat No.*	Blood sugar, mg %	mg P†	Rat No.*	Blood sugar, mg %	mg P†
15a	33	26-50	17a	800	25-45
15b	33	32-49	17b	800	23-38
16	40	26-41	18	1024	23-37
			19	888	23
			20a	812	27-40
			20b	812	27

* and † have the same meaning indicated in Table I.

to provide data for calculating the dry weight of the portion extracted. Aliquots weighing 30 to 80 mg were extracted for 24 hours at 4°C in 5 ml distilled water. The extract used for phosphatase assay was freed of the residue by centrifugation.

Incubation of enzyme with substrate. The substrate consisted of sodium β-glycero-phosphate (concentration 0.5%)[4] at a pH of 8.6 with and without the addition of 0.009 M $MgCl_2$. The incubation of the filtrate with the substrate and the determination of the inorganic phosphorus liberated were carried out according to the procedure described by Shwachman.[4] In the present study, the amount of phosphorus hydrolyzed was related to the dry weight of the tissue.

The average variation in phosphorus liberated by different aliquots of the same enzyme sample for 13 consecutive pairs of samples was 4%; the range for all but one was 0-6%, the exception being 14%. The average variation between the extracts of the 2 kidneys when determined separately was 11.9% for 7 pairs, with a range of 0.8 to 25%. The values of phosphatase activity obtained with added magnesium plotted against the values without magnesium showed a linear relationship, corresponding to the

$$\text{ratio} \frac{\text{activity with Mg}}{\text{activity without Mg}} = 1.7.$$

Histochemical methods employed. After fixation of the tissues in cold 80% alcohol and subsequent imbedding in paraffin, the

4 Shwachman, H., *J. Ped.*, 1941, **19**, 38.

sections were incubated at 37°C in glycerophosphate at pH 9.4 for 3 and 6 hours according to Dempsey and Deane's modification of Gomori's method.[5] Hematoxylin eosin preparations were also made from the same blocks.

Results. The results on the normal and alloxan diabetic rats are presented in Table I, and those on the hypo- and hyperglycemic rats in Table II. The normal rat kidneys released an average of 36 mg P per g of tissue per hour. When the abnormally high results on the kidneys of rat No. 4 were excluded, the average was 28 without Mg, and 47 with Mg. All but one of the alloxan diabetic rats had abnormally low phosphatase activity. Those sacrificed 7 to 12 days after alloxan administration had activities of 5 or less; 3 of the 4 rats allowed recovery periods of 21 to 42 days had activities of 10-13 without Mg, and 14-21 with Mg. Rat No. 11 had a normal phosphatase activity 21 days after the alloxan administration. All of the rats whose blood was analyzed showed marked hyperglycemia.

Neither hypoglycemia nor hyperglycemia, maintained for the relatively short periods of our experiments, had any demonstrable effect on the phosphatase activity of the kidneys (Table II).

There were no qualitative histochemical differences in alkaline glycero-phosphatase between the kidneys of normal and of acutely hyperglycemic and hypoglycemic animals. However, in the rats given alloxan 1 to 6 weeks before autopsy, there was a greatly reduced amount of phosphatase in the epithelium of groups of proximal convoluted tubules in which the cells were flatter and less eosinophilic than average. These tubules were particularly numerous in the subcapsular region and unquestionably represented regenerated tubules that had replaced those damaged by alloxan. In addition, small formless intratubular masses were present in the medulla. These masses were stained by hematoxylin and were frequently covered by epithelium. They appeared to consist of necrotic and calcified clumps of epithelium and were more common in the kidneys of rats killed soon after receiving the dose of alloxan than in rats killed later.

A recent paper[6] reports a drop in the phosphatase content of the kidney in 6 to 72 hours after administration of alloxan, which it ascribes to renal damage. Bennett and Behrens[7] have shown that the elevation in non-protein nitrogen following alloxan is roughly proportional to the dose, is independent of hyperglycemia, is not altered by insulin, and may persist for as long as 31 to 34 days after administration of the drug; and further, that it is correlated with the "histopathological" changes in the kidneys. Breedis, Florey and Furth[8] have reported that, following administration of a nephrotoxic agent, such as uranium nitrate, the reappearance of phosphatase in the regenerating tubules is slow, not attaining a normal value in 25 days. In the present study, 1 of 3 rats exhibited a normal amount of phosphatase after 21 days, a fourth examined after 42 days still showed a low phosphatase activity.

Summary. 1. The alkaline phosphatase activity of the rat kidney, which reflects principally the enzyme content of the proximal convoluted tubules, could not be changed from the normal by varying the amount of glucose for reabsorption over periods of 6 to 12 hours.

2. The kidneys of rats suffering from chronic alloxan diabetes as a rule contained less than normal amounts of alkaline phosphatase. The lowest values were obtained when first examined one week after the dose of alloxan, but recovery of a normal content was not complete when the last examination was performed after 42 days. The proximal convoluted tubule epithelium that had regenerated after the alloxan injury was thus observed to be slow in acquiring its normal enzyme content.

[5] Dempsey, E. W., and Deane, H. W., *J. Cell. Comp. Physiol.*, 1946, **27**, 159.

[6] Menten, M. L., Janouch, M., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 33.

[7] Bennett, L. L., and Behrens, T., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 5.

[8] Breedis, C., Florey, C. M., and Furth, J., *Arch. Path.*, 1943, **36**, 402.

16310

Side Reactions to Pyribenzamine Medication.

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From the University of Iowa.

A number of recent publications have dealt with the use of the new anti-histaminic drugs, and more are appearing every month. Most of these articles mention various side effects encountered, but to date the author has seen no reports of controlled observations of such side effects. The following data regarding pyribenzamine may therefore be of interest.

All the patients who were studied were receiving injections of typhoid vaccine, and the effect of pyribenzamine upon their reactions to the vaccine will be reported elsewhere. At the time of administration of the vaccine, each patient was given either pyribenzamine (PBZ) tablets or indistinguishable placebos,* with instructions as to dosage. Forty-eight adults were on 100 occasions given five 50-mg PBZ tablets with instructions to take one tablet at once, one every 4 hours throughout the day, and one tablet the following morning. Forty-nine subjects were on 102 occasions given placebos with the same instructions. Thirty-seven subjects on 56 occasions were given ten 50-mg tablets of PBZ with instructions to take 2 tablets at a time instead of one. Each subject had a report sheet on which he was asked to indicate whether or not he had experienced any of the symptoms listed on the sheet. The accompanying table shows the percentage occurrence of the symptoms in the 3 groups of subjects.

TABLE I.
Percentage Occurrence of Side Effects.

Symptom	Five 50-mg PBZ tablets in 24 hr (N = 100)	Ten 50-mg PBZ tablets in 24 hr (N = 56)	Placebos, 5 in 24 hr (N = 102)
Drowsiness	37	48	30
Headache	26	36	42
Nausea	17	23	8
Dizziness	24	41	15
Nervousness	13	21	15
Dryness of mouth	29	45	30
Insomnia	12	23	6

* The pyribenzamine and placebos used were supplied by the Ciba Pharmaceutical Company, Summit, N.J.

These data indicate that the smaller dose of PBZ used here, which is the dose most frequently used in treating allergic conditions, has a negligible effect in producing the stated symptoms, all of which have been ascribed to PBZ medication. For example, drowsiness is the most commonly reported side reaction to PBZ, but the administration of 250 mg in 24 hours gave an incidence of drowsiness of only 37% as compared with 30% for placebo medication. The 2 patients who were bitter in their complaints of drowsiness both received placebos. Nausea, dizziness, and insomnia were somewhat more frequent in the 250 mg group as compared with the controls. Headache was less frequent, and nervousness and dryness of the mouth were apparently equally frequent in both groups. Doubling the dose of PBZ was accompanied by a definite increase in the incidence of symptoms, but headache was still less frequent than among the controls.

These data indicate the importance of using controlled studies in evaluating the side reactions, as well as the therapeutic effects, of a new drug.

Summary. A controlled study was made of side reactions to pyribenzamine. Five 50-mg tablets were given over a 24-hour period to 48 subjects; some subjects were used 2 or 3 times, and the total number of times the drug was given was 100. Placebos were given to 49 subjects a total of 102 times. The dosage of pyribenzamine was doubled in 37 other subjects on 56 occasions. Nervousness, dryness of the mouth, and headache were more frequent in the control group as compared with the smaller dose group of pyribenzamine. Drowsiness, nausea, dizzi-

ness and insomnia were less frequent in the control group. All side reactions mentioned occurred more frequently when the dose of pyribenzamine was doubled.

This study was prepared at Walter Reed General Hospital while the author was in the Army Medical Corps.

16311

Protective Action of Vitamins C and P Against Dichlorophenarsine Hydrochloride (Clorarsen).

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This study was undertaken specifically to ascertain whether the use of vitamins C and P in combination would protect against the acutely toxic effect of intravenously injected dichlorophenarsine hydrochloride in mice. It was thought that if these vitamins exerted a favorable effect on capillary fragility it might be demonstrated by the manifestation of a decrease in the acute toxicity of arsenic, since arsenic is a potent capillary poison.

The literature dealing with decreased capillary resistance as one of the toxic manifestations of antisyphilitic therapy has been reviewed by Horne and Scarborough.[1] The apparently protective action of vitamin C against the toxicity of arsenicals has been commented on by several groups of authors.[2,3,4] Scarborough and Stewart have reported that vitamin P (hesperidin) treatment increased capillary resistance in erythema and dermatitis due to arsenic and bismuth.[5]

Goldforb[6] suggested that the vitamin in the form of aqueous extracts of whole lemon might prevent arsenical encephalopathy in patients receiving intensive arsenical therapy. And, Goldstein, Stalman and Goldforb[7] found that treatment with the methyl chalcone of hesperidin decreased the mortality of a standard dose of mepharsen in rabbits from 90 to 57%, a difference that was of questionable significance ($X = 2.9$).

In a preliminary study on groups of control and treated mice (Table I), it was found that vitamin C alone (2.2 millimoles per millimole of dichlorophenophenarsine hydrochloride) had a favorable, but not statistically significant effect. The same was true when similar groups of mice were treated with hesperidin methyl chalcone alone (1 mg each daily for 9 days previous and 3 days following the injection of the arsenical).

For these reasons it was decided to investigate the protective effect of a combination of these two substances on the acute toxicity of a standard dose of intravenously administered dichlorophenarsine hydrochloride.

Method. Adult female white mice, weighing from 17 to 24 g (average weight, 21 g apiece in each group) maintained on a diet of Purina Checkers plus lettuce were used. Injections of dichlorophenarsine hydrochloride (clorarsen, Squibb) dissolved in saline were made into the tail vein, and all animals were observed for 3 days after injection, since those not

1 Horne, G., and Scarborough, H., *Lancet*, 1940, **2**, 66.

2 Sulzberger, M. B., and Oser, L. B., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 716.

3 Bundesen, H. N., Aron, H. C. S., Greenbaum, R. S., Farmer, C. J., and Abt, A. F., *J. Am. Med. Assn.*, 1941, **117**, 1692.

4 McChesney, E. W., Barlow, O. W., and Klinck, G. H., *J. Pharm. and Exp. Therap.*, 1944, **80**, 81.

5 Scarborough, H., and Stewart, C. P., *Lancet*, 1938, **2**, 610.

6 Goldforb, A. E., *Arch. Dermat. and Syph.*, 1941, **43**, 536.

7 Goldstein, D. H., Stalman, A., and Goldforb, A. E., *Science*, 1943, **98**, 245.

TABLE I.

Results Showing the Protective Action of Vitamins P and C Given in Combination Against the Toxicity of Dichlorophenarsine Hydrochloride.

Group No.	Control groups				Group treated with a combination of Vitamins C and P.				Statistical significance
	No. of mice	No. dead	No. survived	% survival	Lot of hesperidine methyl chalcone	No. of mice	No. dead	No. survived	% survival	
I	40	36	4	10	No. 1369	40	7	33	82.5	$X^2 = 39.1$
II	20	17	3	15	" 1369	15	3	12	80.0	$X^2 = 14.7$
Subtotal Lot No. 1369	60	53	7	11.6		55	10	45	81.8	$X^2 = 57.0$
III	16	9	7	44	" 1653	17	0	17	100	$X^2 = 11.9$
IV	20	11	9	45	" 1653	21	8	13	62	$X^2 = 1.17$
V	15	8	7	46.7	" 1653	19	1	18	94.7	$X^2 = 12.7$
Subtotal Lot No. 1653	51	28	23	45.1		57	9	48	84.2	$X^2 = 18.2$
Total	111	81	30	27.0		112	19	93	82.0	$X^2 = 70.6$
	Treated with Vitamin C alone.					Treated with hesperidine methyl chalcone alone				
IA	40	29	11	27.5	No. 1369	40	23	17	42.5	

killed by the arsenical in that length of time were eating well.

In the treated animals, vitamin P was administered as purified methyl chalcone of hesperidin. This substance was kindly made available by Dr. W. Biehm, Medical Director of Abbott Laboratories. When uncertainty existed regarding whether all the arsenical was actually gotten into the vein, the mouse in question was discarded from the series.

The Control Groups. All mice whether in the control or treated groups received a dose of 27 mg of dichlorophenarsine hydrochloride per kilo of body weight. This dose was used because preliminary experiments indicated that it approximated an L. D. 85 dose of the arsenical when injected into the tail vein.

The Treated Groups. These groups received a daily subcutaneous dose of 1 mg apiece of methyl chalcone of hesperidin in saline each day for 9 days previous to and 3 days following the injection of the above dosage of dichlorophenarsine hydrochloride which also contained 2.2 millimoles of ascorbic acid per millimole of dichlorophenarsine hydrochloride. The dosage of ascorbate was chosen for convenience and because the observations of McChesney, Barlow and Klinck[4] have indicated that it confers protection. The dose of vitamin P represented an average dose of 47.6 mg of vitamin per kg of body weight per day and was chosen arbitrarily. It is 36% greater than the dose used by Goldstein and his associates.[7]

Results. The results on the different control and treated groups are shown in Table I. It is to be noted that in each of the 5 groups of tests the treatment provided a significant protection except in the case of Group 4. It should also be noted that there is a difference in the results obtained in Groups I and II, as compared with those in Groups III, IV, and V. This may be attributed to the fact that different lots of both the vitamin and arsenical were used. Unquestionably the second lot of the arsenical was more toxic than the first lot.

Discussion. It is possible that if more mice had been used ascorbic acid alone or hesperidin methyl chalcone alone would have yielded

a statistically significant favorable result. However, a substance to counteract an acutely lethal dose of a substance must act rapidly as well as be potent. On the contrary, Horne and Scarborough found ascorbic acid to be ineffective and hesperidin to be effective in the treatment of a patient with arsenical purpura.[1] Gorrie[8] has also reported hesperidin to be effective in the treatment of arsenical purpura.

Although we were not interested in devising a method for the assay of vitamin P, it is possible that our observations may be sufficiently consistent to be utilized for the development of a method for the assay of vitamin P active substances.

These results would indicate that it would be advisable to provide patients with an abundant source of vitamin P and C for several days prior to and during treatment with arsenicals.

Conclusions. Ascorbic acid and hesperidin methyl chalcone when used as a combined therapy afforded a definite protection in mice against the toxic effect of a single dose of intravenously administered dichlorophenarsine hydrochloride.

[8] Gorrie, D. R., *Lancet*, 1940, **1**, 1005.

16312

Antibacterial Properties of 4-Amino-2-Methyl-l-Naphthol Hydrochloride.*

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As previously reported[1] pyridoxamine acquires antibacterial potency following irradiation at suitable wavelength and pH. Lack of activity in irradiated and non-irradiated solutions of pyridoxine, dimer and trimer of pyridoxine,† pyridoxal and a number of pyridine and benzene derivatives demonstrated that the presence of an amino and one or two hydroxyl substituent groups is an essential prerequisite for the development of antibacterial activity. It was tentatively assumed that the antibacterial factor may be an intermediate product of oxidation.‡ Accordingly, it seemed to be of particular interest to determine the antibacterial potency of 4-amino-2-methyl-1-naphthol hydrochloride (i.e. water soluble derivative of vitamin K, Synkamin of Parke, Davis) in view of the presence of an amino substituent group and the readiness with which the compound undergoes oxidation. The results of the studies are summarized below:

Solutions of Synkamin having the highest antibacterial potency were prepared, as follows:

Tenth normal HCl solution was autoclaved and cooled to 37°C prior to use. Pure dry Synkamin powder§ and sodium bisulfite were dissolved in concentrations of 3 and 0.5 mg per ml, respectively. Immediately after preparation the solution was sealed with vaseline and stored in the refrigerator.

As may be seen from Table I, Synkamin solution prepared as above proved strongly bacteriostatic against gram positive organisms in broth and gram negative bacilli in Gladstone synthetic medium. Among the few strains tested, staphylococcus, strain H and pneumococcus, type I were highly susceptible, while *Staphylococcus hemolyticus* was refractory. As may be also noted from the same

* This investigation has been supported by grants from the Commonwealth Fund and The National Vitamin Foundation.

† The author is thankful to Dr. John V. Scudi for a supply of this compound.

[1] Shwartzman, G., and Fisher, A., *J. Biol. Chem.*, 1947, **167**, 345.

‡ Possibly due to the formation of a lactam ring between the amino and hydroxyl groups following the elimination of a molecule of water.

§ Generously supplied by Dr. L. A. Sweet of Parke, Davis and Company.

TABLE I.
Effect of Synkamin in N/10 HCl Containing 0.05% $NaHSO_3$, Sealed with Vaseline.

Organism tested	Medium	Minimal amount of Synkamin: Complete inhibition	Minimal amount of Synkamin: 25 to 50% inhibition
Staphylococcus, Strain H	Broth	1.5-2 γ	1 γ
Streptococcus hemolyticus, Strain 870	"	—	—
Pneumococcus, Type I	"	2.5 γ	
E. coli, Strain 42	Gladstone synthetic medium	3 γ	2 γ
" " " L	" " "	2.5 γ	1 γ
S. binns	" " "	1.5 γ	0.8 γ
S. paratyphi B.	" " "	2.5 γ	1.5 γ
B. friedlanderi, Type A	" " "	35 γ	
E. coli, Strain 42	Broth	—	—
S. binns	"	—	—
Meningococcus, Group I	Synthetic medium[2]	0.75 γ	
" " "	Broth	2.5-3 γ	

— = No inhibition with 100 γ of Synkamin.

The composition of Gladstone synthetic medium and methods of determination of bacteriostatic activity and the size of inocula were the same as previously described.[1]

table and additional experiments not recorded in the table, gram negative bacilli (with the exception of *B. friedlanderi*, type A.) were markedly susceptible to Synkamin. All the gram negative bacilli tested were resistant to the compound in broth, suggesting the presence of an antagonistic factor in this medium. The antagonistic effect of broth was less pronounced with meningococcus, type I, the organism being approximately only 4 times more susceptible in synthetic medium than in broth. In addition to broth, casein hydrolysate and blood serum proved antagonistic to the activity of Synkamin against *E. coli*. Various concentrations of the substances were added to Gladstone synthetic medium containing different amounts of Synkamin. The mixtures were inoculated with *E. coli*, strain 42. There was obtained 50% reversal of the antibacterial activity in the following ratios of dry weights, namely, one part of Synkamin to 125 parts of casein hydrolysate, 65 parts of mouse serum and 50 parts of rabbit serum. It remains as yet unknown whether staphylococcus would show greater susceptibility in synthetic medium than in broth. No reversal of antibacterial activity was noted following the addition of casein hydrolysate and serum to broth cultures of staphylococcus, strain H. The nature of the factor antagonizing inhibition of *E. coli* was not yet identified.

The experiments about to be described were done in order to determine the effect of oxidation by air upon the antibacterial activity of Synkamin. The preparations were tested against *E. coli* in Gladstone synthetic medium and staphylococcus, strain H in broth. Solutions of Synkamin in N/10 HCl, in concentration of 3 mg per ml with and without vaseline seal were stored in the dark at 4°C. Twenty-four to 48 hours following preparation there occurred a 25% loss of activity in the non-sealed preparation. The titer remained stationary during the following 6 days. There was almost complete loss of activity 10 days following preparation. In the vaseline sealed preparations the titer was unchanged for the entire period of observation, i.e. 3 to 4 weeks.

Immediately upon preparation solutions containing 3 mg of Synkamin and 1 mg of sodium bisulfite in N/10 HCl gave complete inhibition of *E. coli* in concentration of 2-3 γ of Synkamin per ml of synthetic medium. On exposure to air for 4 days at 4°C there occurred a 25% loss of activity. On the other hand, addition of 2 mg of sodium bisulfite to 3 mg of Synkamin produced an immediate 40% decrease of activity which was completely restored on air exposure at 4°C for 4 days. The effect of the reducing substance upon the activity of Synkamin against staphylococcus, strain H was not as clear-cut, possibly in view of the interference of broth with the reduction.

[2] Frantz, I. D., *J. Bact.*, 1942, **43**, 757.

Additional experiments are under progress in order to investigate this relationship.

Additions of cystine, 1 mg, methionine, 3 mg, and methionine sulfoxide,‖ 5 mg, to 3 mg of Synkamin in N/10 HCl, per ml failed to antagonize the effect of Synkamin upon *E. coli,* strain 42 and staphylococcus, strain H.

It is obvious from the above experiments that the antibacterial activity of Synkamin described is intimately related to air oxidation. Excessive oxidation brings about loss of activity. Reduction with sodium bisulfite results in reversible decrease in potency which is restored on exposure to air. It is suggestive that the antibacterial activity of Synkamin is due to an intermediate state of oxidation by air which possibly takes place immediately upon dissolving the substance. The antibacterial potency can be maintained at a constant level by timely exclusion of air from an oxidized solution or by additional oxidation of a reduced solution.

The effect of Synkamin was compared with that of 2 other water soluble derivatives of vitamin K, sodium-2-methyl-1,4-naphthoquinone diphosphate (Synkayvite, Hoffmann-La Roche) and 2-methyl-1,4-naphtho-hydroquinone-3-sodium-sulfonate (Hykinone, Abbott).¶ The solutions were made similarly to Synkamin, with and without vaseline seal, and tested against staphylococcus, strain H in broth and *E. coli,* strain 42 in Gladstone synthetic medium. All solutions of Synkayvite were totally devoid of antibacterial activity against these organisms in concentrations 1-200 γ per ml. Immediately following preparation and following storage at 4°C, Hykinone showed no effect upon *E. coli.* After one week storage at room temperature the compound inhibited *E. coli* in the concentration of 40 γ per ml of synthetic medium. Staphylococcus, strain H, was completely inhibited by 10 γ and partially inhibited by 5 γ per ml of broth. Thus, Synkayvite possessed no activity under conditions described, while the potency of Hykinone was distinctly lower than that of Synkamin. The antibacterial effect also seems to be significantly greater with Synkamin than with vitamin K derivatives studied by previous authors,** i.e. 2-methyl-1,4-naphthoquinone and derivatives containing chloro, methyl and methoxy substituent groups.

Summary. The points of interest of the studies embodied in this note are as follows: 4-amino-2-methyl-1-naphthol hydrochloride possesses marked antibacterial activity against gram positive and gram negative organisms.

The compound is strongly effective against gram positive organisms in presence of broth, casein hydrolysate and blood serum, and against gram negative organisms in synthetic medium. The activity against the latter organisms is antagonized in broth medium. Casein hydrolysate and mouse and rabbit sera antagonize the antibacterial activity of Synkamin against *E. coli* in a significantly lesser degree than broth, namely, in dry weight ratios of 125:1, 65:1, 50:1, respectively.

The presence of an amino substituent group and partial oxidation of the substance under carefully controlled conditions play an important role in the antibacterial activity described.

The author is indebted to Miss Alice Fisher for capable and accurate assistance.

‖ Kindly made available by Dr. Joseph Seifter of Wyeth Institute.

¶ Thanks are due to Dr. Elmer L. Sevringhaus of Hoffmann-LaRoche, Inc., for a supply of Synkayvite and to Dr. Arnold E. Osterberg of Abbott Laboratories for a supply of Hykinone.

** No detailed comparison can be presented in this brief communication. Reports of previous investigations are listed under References.[3]

[3] Fosdick, L. S., Fancher, O. E., and Calandra, I., *Science,* 1942, **96**, 45; Armstrong, W. D., Spink, W. W., and Kahnke, I., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 230; Wooley, D. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 225; Colwell, A., and McCall, M., *J. Bact.,* 1946, **51**, 659; and Hoffmann-Ostenhof, O., *Science,* 1947, **107**, 549.

16313

Protection of *Escherichia coli* against Bacteriophage with Citrus Pectin.

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Since the discovery in this laboratory[1,2] that apple pectin and certain other polysaccharides inhibit the hemagglutinating activity and the multiplication of influenza A virus it became desirable to determine whether other viruses as well could be combatted with such polysaccharides. A suitable bacteriophage acting on *Escherichia coli* suggested itself as a useful system for this purpose, not only because of the facility with which various aspects of the virus-host relationship might be studied, but also because circumstantial evidence has pointed to the conclusion that the point of viral attack on the bacterium is carbohydrate in nature.[3] This latter point was attractive because the discovery of the effect of apple pectin in inhibiting influenza virus was based on belief in the polysaccharide nature of the cell receptor of this virus.[1,2] Just as in the case of influenza A virus, so with bacteriophage the host cells might be protected by supplying an antagonistic polysaccharide to compete with the receptor substance. For these reasons pectins were examined for ability to protect the bacterium from the phage, and it was found that citrus pectin was capable of doing this.

To demonstrate this phenomenon, the T_2 phage and its susceptible host strain of *Escherichia coli* were best suited.† Inocula for the tests were prepared by incubating the strain of *E. coli* in a synthetic medium[4] for 2.5 hours at 37°C. At this time growth was just plainly visible. 0.5 cc of this fresh young culture was used to seed each tube of the synthetic basal medium of Hook *et al.*[5] which was used in all of the tests, unless otherwise noted. The inoculum of T_2 bacteriophage was prepared by growing the virus in the susceptible strain of bacteria cultivated in Todd-Hewitt broth[6] for 20 hours at 37°C. The lysate was filtered through a Seitz pad to provide cell-free material and the filtrate was titered by the plaque count method.[7] Suitable dilutions were then used in the tests. The same phage filtrate was used throughout this investigation, and was maintained potent by storage at 4°C. Solutions of citrus pectin were prepared exactly as described previously,[2] and no other manner of obtaining solutions was found satisfactory.

When the synthetic basal medium of Hook *et al.* fortified with 100 mg of citrus pectin

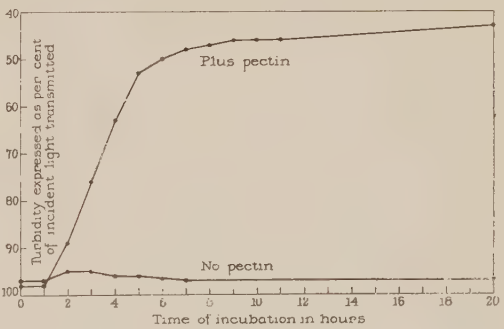

Fig. 1.

Effect of pectin on the growth curves of *E. coli* in the presence of T_2 bacteriophage.

* Major, Veterinary Corps, U. S. Army.

† We wish to thank Dr. S. E. Luria for transplants of these organisms.

1 Woolley, D. W., and Green, R. H., *J. Bact.*, 1947, **54**, 63.

2 Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.

3 Burnet, F. M., Koegh, E. V., and Lush, D., *Australian J. Exp. Biol. and Med. Sci.*, 1937, **15**, 227.

4 Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

5 Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165**, 241.

6 Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35**, 973.

7 Hershey, A. D., Kalmanson, G., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 267.

TABLE I.
Influence of Citrus Pectin on Multiplication of *E. coli* and T_2 Bacteriophage. Counts Made After 20 Hours Incubation at 37°C.

Additions to basal medium	*E. coli* cells per cc	Phage particles per cc
Bacteria	1×10^9	
" + pectin	1×10^9	
" + phage	0	11×10^9
" + pectin + phage	2×10^9	5×10^9

per 6 cc was inoculated with 10^8 cells and then with the 10^9 phage particles, and the mixture was incubated at 37° overnight, heavy growth of the bacteria occurred. In contrast, when no pectin was added, no bacterial growth was visible. In the absence of pectin the virus had acted in its usual fashion and had lysed the host. Furthermore, the citrus pectin was not injurious to the growth of the bacteria in the absence of phage, because multiplication of coli in tubes of medium plus pectin was equal to that in the basal medium without pectin. Growth curves of *E. coli* in the presence of the T_2 phage, with and without citrus pectin are shown in Fig. 1. Quantitative data on the numbers of viable cells in experiments such as these will be found in Table I.

The concentration of citrus pectin needed to effect this protection of the bacterial cells was determined by adding graded amounts of the substance to the basal medium which was subsequently inoculated with *E. coli* and with T_2 phage, and incubated for 20 hours. Sixty mg of citrus pectin per 6 cc of culture was the smallest quantity which would allow good growth of the bacteria.

A number of polysaccharides were tested for ability to protect against the phage, and citrus pectin was found to be the most effective. Apple pectin at 125 mg per 6 cc would not allow growth of the bacteria in the presence of the virus when the incubation was for 20 hours, but in a test of 40 hours' duration this substance was almost as effective as citrus pectin. Gum acacia (125 mg per 6 cc‡) or starch (125 mg per 6 cc) were inactive. This order of potency was not the same as that for these substances acting against influenza virus. In that instance Green and Woolley[2] found that apple pectin was more active than citrus pectin in causing inhibition of multiplication of the virus, and that gum acacia had some potency. All 3 substances, but not starch, were inhibitors of hemagglutination caused by the virus.

The power of citrus pectin to enable *E. coli* to grow luxuriantly in the presence of its bacteriophage is the basic observation in this work. The experiments which are now to be outlined were performed in an effort to learn how this action was accomplished.

It was first necessary to ascertain whether the action of citrus pectin was attributable to the increased viscosity produced by it in the medium. Work of earlier investigators has led to the conclusion[8] that substances which made the medium highly viscous protected bacterial cells from destruction by phage. This old opinion proved not to be entirely correct, because in the present work it was found that viscosity and protective action against the virus were not always correlated. Potent protective substances with very low viscosity, as well as inactive materials of high viscosity were both found. As D'Herelle has stated many viscous substances exert protective action against lysis, but this correlation proved to be illusory because non-viscid material which was still quite active was found. Thus, a viscous material such as gelatin at 60 mg per cc of the culture fluid allowed good growth of *E. coli* in the presence of phage, and in this respect resembled citrus pectin. Both media were viscid (time of flow of the gelatin fortified basal medium 524 seconds in an Ostwold viscosimeter at 37°C and of the pectin containing medium 259 seconds). However, when the gelatin was digested with crystalline trypsin overnight,

‡ Subsequent experiments showed that 240 mg of acacia per 6 cc did exert some effect.

8 D'Herelle, F., *The Bacteriophage and Its Behavior,* Williams and Wilkins Co., 1926.

TABLE II.
Influence of Citrus Pectin on Survival of Cells in Presence of Bacteriophage as Shown by Plate Counts Made 15 Minutes After Mixing the Participants.

Additions to basal medium	Numbers of organisms after 15 min.	
	$10^2 \times$ *E. coli* cells per cc	$10^8 \times$ phage particles per cc
Phage		3
Bacteria	4,000,000	
" + pectin	5,000,000	
" + phage	3	1
" + pectin + phage	3,000,000	3

and the digest was dialyzed against running water, the nondialyzable portion retained full (and even slightly enhanced) powers of protection against lysis by the phage. Digestion so reduced the viscosity that basal medium containing the preparation at a level equivalent to 60 mg of gelatin per cc was scarcely more viscous than the basal medium without any additions (time of flow 99 seconds compared to 84 seconds). Possibly the polysaccharide portion of gelatin which remained after the proteolysis, was responsible for the protective effect against the phage. The lack of correlation of viscosity and protection from lysis may be demonstrated in the reverse manner. Thus, the basal medium fortified with Tween 80 (125 mg per cc) at pH 5.7, was more viscous than that containing an effective concentration of citrus pectin. Nevertheless, the Tween did not prevent lysis of the bacteria by the virus. Therefore, the protection afforded by citrus pectin was not due solely to the fact that it increased the viscosity of the medium.

The effect of the pectin was not to destroy the phage, because no virucidal power could be demonstrated. Thus, when 10^{10} phage particles were added to citrus pectin solution (20 mg per cc), and the mixture incubated for 30 minutes, diluted and assayed by the plaque method, 10^{10} particles were found.

The data of Table I show that while citrus pectin allowed the bacteria to grow in the presence of the phage, it did not prevent multiplication of the virus. Thus, nearly as many virus particles were found in 20-hour cultures containing abundant viable bacterial cells supported by pectin, as in the control cultures without pectin where few cells survived. This is exactly the situation which obtains with lysogenic strains[9] in which one finds growth of the host strain, and no lysis, concomitant with multiplication of the phage. Therefore, the addition of citrus pectin to the system of *E. coli* and T_2 phage has reproduced the phenomenon of lysogenic strains. This change was dependent on the continued presence of the pectin.

Citrus pectin did not prevent the attachment of the virus to the host cell, but it did protect the cell from lysis, *i.e.*, destruction. This protection from lysis may be seen from the results of the following experiments. Two tubes of basal medium, one containing pectin (100 mg per 6 cc), and the other none, were inoculated with approximately 10^8 young cells and 10^8 phage particles and the mixtures were incubated at 37°C for 15 minutes. During this time no multiplication of the bacteria would be expected. Dilutions were made, and in these the numbers of viable cells and of phage particles were determined by the usual plate counts. The results of a typical run are shown in Table II, where it can be seen that in the tube without pectin most of the viable cells had become infected and subsequently disappeared through lysis while in the tube with pectin no significant decrease had occurred.

The fact that pectin did not prevent attachment of the phage to the cells was shown in the following way. The experiment immediately preceding was repeated, except that after the 15-minute incubation period the cells were collected by centrifugation and washed 4 times with a solution of citrus pectin (20 mg per cc). Dilutions were then made in water, and the numbers of viable cells and of phage

[9] Northrop, J. H., *J. Gen. Physiol.*, 1939, **23**, 59.

particles were determined. The object of the pectin washing was to remove any virus particles which were not attached to cells. The results showed that in the tube which contained pectin when the host and parasite were mixed, 2 x 10^8 phage particles, the number actually introduced as inoculum, still remained. These must therefore have been fixed to the cells in a manner firm enough not to be dislodged by washing. The results were similar when water or physiological saline were used as wash. However, the pectin solution was used as wash so that excess phage could be removed without danger of subsequent infection of the cells. If water had been used, the pectin in the culture would have been diluted and thus rendered impotent before the excess of virus had been eliminated. Infection of the cells subsequent to the main experiment might thus have occurred.

From the above experiments it seemed probable that the pectin formed a protective layer around the bacterial cells. This layer did not prevent the attachment of the virus to the host; but it did preclude destruction of the cells. Furthermore, it did not prevent the multiplication of the virus. Moreover, this protective influence of the pectin remained with the cell during dilution of the culture and subsequent plating on agar. This fact is well illustrated by the data in line 5 of Table II.

An apparently therapeutic effect of citrus pectin on cells previously infected with the phage was observed, but this was shown to be spurious, and to have resulted from a very few uninfected cells among the infected ones. Special means were required to detect these uninfected cells, because by the ordinary plating methods none could be found. Thus, a suspension of 10^8 fresh young cells was mixed with 10^9 phage particles and the whole was incubated for 15 minutes. The cells were then collected by centrifugation and washed to remove excess virus. They were then used as inoculum for two tubes, one containing basal medium and the other basal medium plus citrus pectin. The tubes were incubated at 37° for 20 hours, and it was then found that the one with pectin contained luxuriant growth, while the one without had none. Plate counts had been made on dilutions of the inoculum of infected cells, and no living bacteria had been detected. However, when the counting was done on plates made from pectin-containing basal medium (solidified with agar) a few (43) viable, and therefore presumably uninfected cells were found. One may conclude that the presence of the pectin in the plating medium protected those uninfected cells from attack by the large amounts of virus liberated from their infected neighbors, and thus allowed detection. The probable explanation of the phenomenon observed in the tubes now seemed clear. The few cells which escaped infection during the period of exposure to phage were thereafter protected from the phage by the addition of pectin and proceeded to multiply.

The protection of bacterial cells from destruction by bacteriophage was not an isolated observation confined to one strain of the organisms employed. The same relationships were found with *E. coli* strain No. 8677 of the American Type Culture Collection in combination with either the T_2 phage, or with phage strain 8677 of the A.T.C.C. This bacterium, however, was less well adapted for the experiment than was the Luria strain, because when incubation of it with the phage was continued for 20 hours or longer, overgrowth of the previously lysed culture occurred. The effect of pectin, therefore, could be observed for a much shorter time.

One additional fact was, however, established with the A.T.C.C. organisms, namely, that the amount of pectin needed for protection was practically independent of the number of phage particles introduced. The amount of phage was varied over a 100-fold range without altering the influence of a minimal effective amount of pectin.

The discovery of the inhibiting action of apple pectin and certain other polysaccharides on influenza virus[1,2] arose from a working hypothesis which postulated a specific polysaccharide in the host cell which the virus attacked. This receptor substance was postulated to compete with pectin for the attention of the virus because of structural analogy of the two. Woolley (in press) has pursued this working hypothesis further, and has purified

from erythrocytes of species susceptible to the virus, a polysaccharidelike substance which does compete with apple pectin for the virus acting as a hemagglutinin. Therefore, a search for a substance in *E. coli* which might antagonize the protective action of citrus pectin on this species seemed advisable. A number of attempts to demonstrate such a substance have been made, but none has succeeded. The testing procedure employed for this was to add the various samples to the basal medium plus citrus pectin (100 mg per 6 cc) and then to inoculate with 10^8 young cells and with 10^9 phage particles. Incubation of the mixtures for 20 hours should reveal an active preparation by failure of the bacteria to grow. This should follow logically because if the pectin were competing with a cell receptor, increasing the total concentration of the latter in the system should nullify the action of the pectin. The concentration of this receptor in the system is fixed by the number of bacteria, but it might be increased by adding extracts of the substance from the cells. Extracts were prepared from 20-hour cultures of *E. coli* by treating the cells with weak alkali, by grinding them with sand, by heating to 70° or 100°, by ultraviolet light irradiation, and by plasmolysis with ether or with chloroform. None was found able to overcome the antiviral action of citrus pectin. Needless to say, the influenza virus receptor substance extracted from human erythrocytes likewise was ineffective.

Summary. Citrus pectin was found to allow growth of *E. coli* in the presence of an excess of bacteriophage. The pectin was nontoxic to the bacteria, and was not virucidal. The action of the pectin was found not to be the prevention of attachment of the virus to the cell, and indeed, it did not inhibit the multiplication of the phage appreciably. It did, however, protect the cells from lysis, and hence in its presence a phenomenon similar to that seen with lysogenic strains was observed. Gum acacia and starch, representatives of other classes of polysaccharides, were ineffective. No substance could be demonstrated in the cells which was antagonistic to citrus pectin in the way that an influenza virus substrate in erythrocytes was antagonistic to apple pectin.

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Effect of Thyro-Parathyroidectomy upon the Blood and Plasma Volumes of the Rat.

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There have been few studies of blood and plasma volumes in myxedema or experimental hypothyroidism, none on the rat. Thompson[1] in an early study found the plasma volume in myxedematous patients to be 30% below normal, and also found that the values returned to normal upon treatment with thyroid extract. Holböll[2] likewise found the blood and plasma volume reduced in myxedema. In the most recent study, Gibson and Harris[3] studied 7 myxedematous patients. They found that

* The authors gratefully acknowledge the technical assistance of W. Lew and W. Wong. Hemoglobin solution was provided through the courtesy of Dr. Robert B. Pennell, Sharp & Dohme, Inc., Philadelphia, Pa. This study was supported by a grant from the U. S. Public Health Service. Dr. Lippman is now at the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles.

1 Thompson, W. O., *J. Clin. Invest.*, 1926, **2**, 477.

2 Holböll, S. A., *Acta med. Scandinav.*, 1930, **73**, 538.

3 Gibson, J. G., 2nd, and Harris, A. W., *J. Clin. Invest.*, 1939, **18**, 59.

TABLE I.

Effect of Thyro-parathyroidectomy upon the Blood and Plasma Volumes of the Rat.

Final wt, g	Heart wt, g	Wintrobe hematocrit	Blood Vol. Exper., cc	Blood Vol. Norm., cc	Blood Vol. % Norm.*	Plasma Vol. Exper., cc	Plasma Vol. Norm., cc	Plasma Vol. % Norm.	Erythrocyte Vol. Exper., cc	Erythrocyte Vol. Norm., cc	Erythrocyte Vol. % Norm.
180	650	44.5	11.3	12.3	92	6.0	7.0	86	5.3	5.4	98
150	490	43.0	9.8	10.7	92	5.4	6.1	90	4.4	4.6	97
140	481	36.5	8.2	10.2	80	5.0	5.8	86	3.2	4.4	73
127	488					4.1	5.4	76			
120	510	35.0	9.4	9.1	103	5.9	5.2	113	3.5	3.9	89
159	500	41.0	9.6	11.2	86	5.5	6.4	86	4.1	4.8	86
156	553	39.0	10.5	11.1	94	6.2	6.3	98	4.3	4.8	89
174	546	37.0	10.6	12.0	88	6.5	6.8	96	4.1	5.2	79
206	620					6.9	7.6	91			
186	604	39.5	11.5	12.6	91	6.8	7.1	96	4.8	5.5	86
186	651	44.5	11.0	12.6	87	6.3	7.1	89	4.8	5.5	87
238	856	44.0	15.4	15.1	102	8.4	8.5	99	7.0	6.6	106
188	570	41.0	12.0	12.7	94	6.9	7.2	96	5.1	5.5	93
202	610	41.5	11.8	13.4	88	6.7	7.5	89	5.1	5.9	87
148	478	38.0	9.4	10.6	89	5.6	6.0	93	3.7	4.6	80
176	520	38.0	11.8	12.0	98	7.1	6.8	104	4.7	5.2	90
Mean					92			93			89
					± 1.6			± 2.13			± 2.24

* The normal blood volume, considered as 100%, has a standard deviation of the mean of 1.38%. The normal plasma volume has a standard deviation of the mean of 1.6%. The standard deviation of the differences, computed from these data, indicate that the differences are significant.

the blood volume was only 15% below normal, and attributed the lower results of earlier authors to errors in the method of blood volume determination used.

Methods. This study utilized 16 male rats, ranging in weight from 150 to 180 g at the start. Thyro-parathyroidectomy was performed, under ether anesthesia, by blunt dissection and cauterization of the thyroid bed. Following this, an interval of 3 weeks elapsed, during which the rats were undisturbed and were fed upon stock diet. At the end of 3 weeks, the rats had failed to gain weight in accordance with the normal growth curve. Blood and plasma volume determinations were performed according to the hemoglobin dye method previously described.[4] Gross postmortem examination showed no regeneration of thyroid tissue at the site of operation. The heart weights were diminished in accordance with previous experience upon thyroparathyroidectomized rats.[5]

Results. Plasma volumes were obtained in 16 rats, and blood volumes were obtained in 14 of these. There was a mean reduction of plasma volume, erythrocyte volume and total blood volume of approximately 10%, when compared with normal standards for rats of the same weight, as previously established.[4] (Table I).

[4] Lippman, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 188.

[5] Addis, T., Karnofsky, D., Lew, W., and Poo, L. J., *J. Biol. Chem.*, 1938, **124**, 33.

16315

Protective Action of Dietary Cholesterol in Experimental Thyrotoxicosis.*

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Experiments were made to investigate further the well known effects of hyperthyroidism on cholesterol metabolism.[1] An unexpected result of these studies was the observation that a reverse relationship also existed, *i.e.* that high dietary cholesterol modified the toxic effects of high doses of thyroid hormone, in particular, as far as survival time and body weight changes were concerned. These findings are reported in the present communication.

Experimental Procedure. Normal rats were fed *ad libitum* this laboratory's stock diet, slightly modified to contain more yeast, in order to assure an adequate supply of the vitamin B complex.† In addition, thyroid powder was mixed with the diet at concentrations corresponding to 0.4-0.8% thyroid U.S.P. One group of rats (T) received the high thyroid diet as described, a second group (T + C) was fed an identical diet, except that it contained in addition, 1% cholesterol and 0.5% bile salt. The cholesterol was dissolved in cottonseed oil, replacing a corresponding amount of the oil in the diet. The bile salt was added to increase absorption of cholesterol.[2] The rats were about 9 months (Experiment 1) and 38 days (Experiment 2) old respectively at onset of the feeding periods. Litter mates were distributed evenly between the two groups. Body weights were measured once weekly, and the survival time

* Supported by a grant from the Life Insurance Medical Research Fund.

[1] Peters, J. P., and van Slyke, D. D., *Quant. Clinical Chem., Interpretations I*, 2nd Edition, 1946, 497.

† Modified stock diet: 328 g whole wheat flour; 327 g ground oats; 100 g skimmed milk powder; 30 g alfalfa; 95 g yeast, Anheuser-Busch strain G; 80 g cottonseed oil; 20 g fortified cottonseed oil, containing 2500 I.U. vitamin A/g and 400 I.U. vitamin D_2/g; 5 g NaCl; 5 g $CaCO_3$.

[2] Schoenheimer, R., *Biochem. Z.*, 1924, **147**, 258.

TABLE I.
Effect of High Dietary Cholesterol on Survival Time of Rats Fed Toxic Doses of Thyroid Hormone.

Exp. No.	Diet	No. of rats	Age at onset	Mean survival in days	Standard error of mean*	$\frac{D_M}{S_{D_M}}$†
1	T	6	9 mos.	23	± 4.3	13.4
	T + C	6	9 "	55+	0	
2	T	14	38 days	86	± 3.9	3.8
	T + C	7	38 "	111+	± 5.4	

* The standard error of the mean calculated from the formula $S_M = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$ where "d" is the deviation from the mean, and "n" is the number of observations.

† Ratio, difference of means (D_M) to standard error of difference (S_{D_M}); results are considered significant when this ratio > 3.

$$S_{D_M} = \sqrt{\frac{\Sigma d_1^2 + \Sigma d_2^2}{(n_1 - 1)\ (n_2 - 1)} : \frac{n_1 n_2}{n_1 + n_2}}$$

was noted. In the second experiment, the food intake was also measured, the animals being kept in single cages with screen bottoms.

Results. The experimental results are summarized in Table I and Fig. 1. In the first experiment, in which older rats and a desiccated hog thyroid preparation containing 0.65% total I and 0.31% thyroxine were used, the high dietary cholesterol had striking effects, both appreciably prolonging the length of survival time and protecting against the loss in body weight. All rats of group T died within 5-36 days, the average survival time being 23 days. In group T + C, all animals were alive at 55 days, at which time the animals were killed for autopsy. Fig. 1 indicates that the excessive weight loss caused by thyroid hormone (group T) also was prevented or diminished by the addition of cholesterol + bile salt (group T + C).

In Experiment 2 in which young rats and a different hormone preparation‡ were used, the same trend was observed, but the protective action of cholesterol was less marked. The difference in the mean survival times between the groups T and T + C was still significant. The body weight results were equivocal, however; only in the case of the male rats was a similar trend observed as in Experiment 1.

The possibility was considered that the animals on the high cholesterol diet (T + C) ate less due to the presence of bitter-tasting bile salt in their food. In this case the amount of thyroid hormone consumed by this group would have been correspondingly smaller. In order to investigate this possibility, the food consumption was measured in Experiment 2. The results indicate that the animals ate practically the same amount of food in both groups. The average intake per day was 17 g in group T, and 16 g in group T + C. It is highly improbable that such an insignificant difference in the amount of food and thyroid hormone consumed was responsible for the marked differences in survival time and body weight observed. The latter differences have to be attributed, therefore, to an action of the cholesterol protecting from the toxic effects of thyroid hormone.

This conclusion was confirmed by experiments made recently in collaboration with Dr. B. Ershoff.[3] In those experiments, conditions were essentially the same,§ except that bile salt was entirely omitted from the diet. It can be assumed that under these con-

‡ Thyroid powder, U.S.P. Armour.

[3] Ershoff, B. H., and Marx, W., to be published.

§ A different strain of rats and a different stock diet were used; the cholesterol was mixed with the diet as a dry powder (for group T + C).

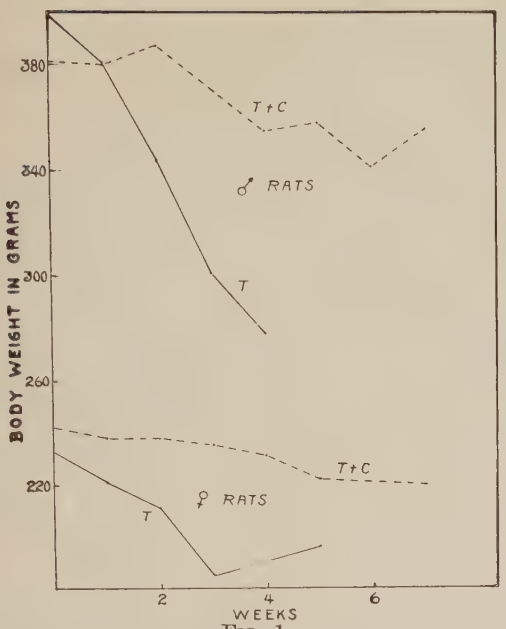

FIG. 1.

Effect of dietary cholesterol on the body weight of rats fed toxic doses of thyroid hormone. T = thyroid alone; T + C = thyroid + cholesterol.

ditions both groups T and T + C consumed the same amount of diet, and therefore, of thyroid hormone. It is interesting that, in this experiment, the survival time was again significantly prolonged as a consequence of the high dietary cholesterol, in spite of the fact that the absorption of the latter was very likely smaller than in the experiments discussed above.

Discussion. The authors are not aware of any report in the literature referring to experimental observations on a protective effect of high dietary cholesterol in case of thyrotoxicosis. The findings are in agreement, however, with a hypothesis recently proposed by Hoffmann and Hoffmann.[4] According to this theory, thyroid hormone stimulates the enzymatic breakdown of lecithin to lysolecithin. Lysolecithin in turn, is believed to affect the central nervous system, to influence cell permeability, and to exert, at higher concentrations, "toxic effects in various organs." These are the effects which cholesterol is supposed to counteract, or to neutralize. It might be added, in connection with this concept, that an antagonism between phospholipids and cholesterol was postulated by various investigators, and many instances can be quoted where such an antagonism was demonstrated experimentally.[5]

Summary. High dietary cholesterol prolonged significantly the survival time of rats fed toxic doses of thyroid hormone; in some, but not in all groups the animals' weight loss was also reduced.

We are very grateful to Mr. William C. Werkheiser of this department for determinations of iodine and thyroxine on a sample of desiccated thyroid, and to Dr. Frederic Fenger of the Armour Laboratories, and to Mr. Noble F. Payton of the Suburban Chemical Co., Chicago, for the cholesterol used in this work.

[4] Hoffmann, F., and Hoffmann, E. J. de, *Public. del Institut. Fisiol.*, Universidad de Chile, 1943; quoted by Foldes, F. F., and Murphy, A. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 218.

[5] Bills, C. E., *Physiol. Rev.*, 1935, **15**, 1.

16316

Toxicity and Pharmacology of SN 13592.* An Analogue of Phenyl Pantothenone.

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The oral antimalarial activity of phenyl pantothenone[1] led to the preparation of compounds which might be considered as thio analogues.[2] One of these was SN 13592,[3] (-)-α,γ-dihydroxy-β,β-dimethyl-N-(2-(phenyl-mercapto-)-ethyl)-butyramide. This compound was examined for trichomonicidal activity.[4] Since high *in vitro* activity was demonstrated, parallel studies of toxicity and systemic effects of this substance were conducted, as representative of the type of phenomena which might be shown by a series of similar phenyl pantothenone analogues. The toxicity tests, for the most part, were based on those described in the *Survey of Antimalarial Drugs*,[3] with minor modifications. The tests are referred to by the designations given them in that publication.

Acute toxicity. Pantothenic acid has an extremely low oral toxicity for the rat. The lethal dose is greater than 10 g/kg.[5] SN 13592 is characterized by a similar low toxicity, being of the order of 5 g/kg. The administration of larger doses by means of the stomach tube was difficult because of the low solubility of the compound. Death at toxic levels of calcium pantothenate is the result of respiratory failure, although lower doses do not seem to affect the respiratory system.[6] With SN 13592, all doses above 500 mg/kg were attended by marked respiratory difficulty, which usually disappeared within 1-2 hours in surviving animals. This respiratory effect was the subject of further study, as described under "systemic effects."

While acute intravenous toxicity was not investigated, it was noted that in one case a dog was infused with a total of 2.6 g (397 mg/kg) over a period of 5 hours without fatal result.

Chronic toxicity. Tests were performed to determine the chronic toxicity for several species of animals. An 11-day rat test was conducted according to the procedure of Test 1-U,[3] with the deviations in weight gains between experimental and control animals as the criterion. During the experimental period, groups of rats receiving 1/16, 1/8, or 3/16 of the lethal dose daily averaged a weight gain similar to that of control rats fed the vehicle alone, and also similar to that of control rats fed the same fraction of the lethal dose of a reference compound, quinine. As shown in Table I, there were a few deaths, but it is doubtful whether these were attributable to the drugs since they also occur with the reference drug.

Histological examination of major organs of these rats, including the intestinal tract, revealed no significant pathological changes.

A 7-day mouse test (Test 1-B) gave similar results as far as weight gains were concerned. Groups at 45, 90, and 270 mg/kg/day exhibited average weight gains comparable to the control animals, but mortality was much higher at daily doses greater than 270 mg/kg.

For studies with the chick Test 1-A was used. The maximum tolerated dose (that daily dose at which the final weight is approximately equal to the starting weight) was determined to be 4000 mg/kg/day for the 4 days of the test. However, at 500 mg/kg/day the chicks gained as much weight as the controls, and as much as the group fed 50/kg/day of the reference drug quinine. It is interesting to note the variation in weight

* The Survey Number, SN 13592, designates this compound in the *Survey of Antimalarial Drugs.*

1 Woolley, D. W., and Collyer, M. L., *J. Biol. Chem.*, 1945, **159**, 263.

2 Senear, A. E., Rapport, M. M., and Koepfli, J. B., *J. Biol. Chem.*, 1947, **167**, 229.

3 Wiselogle, F. Y., *A Survey of Antimalarial Drugs*, Edwards, Ann Arbor, 1946.

4 Johnson, G., and Kupferberg, A. B., in press.

5 Molitor, H., *Fed. Proc.*, 1942, **1**, 309.

6 Unna, K., and Greslin, G., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 311.

TABLE I.
Chronic Toxicity of SN 13592—Test 1-U.

Group	No. of rats	Compound	Fraction of lethal dose daily	Total mg/kg (11 days)	No. of animals surviving	Avg wt gain (g/11 days)
A	6	Control	—	—	6	49.8
B	6	Quinine	1/16	308	5	45.4
C	6	”	1/8	616	6	50.0
D	6	”	3/16	924	6	49.0
E	5	SN 13592	1/16	3432	4	42.5
F	6	”	1/8	6875	6	60.3
G	6	”	3/16	10307	5	40.0

TABLE II.
Chronic Toxicity of SN 13592—Test 1-A. Five chicks in each group.

Group	Compound	Dose mg/kg/day	No. of chicks surviving	Avg wt change (g/4 days)
A	Vehicle	—	5	+22.4
B	Quinine	350	4	+16.2
C	”	200	5	+25.8
D	”	100	5	+25.8
E	”	50	5	+28.0
F	SN 13592	4000	4	— 1.2
G	”	2000	4	+11.0
H	”	500	5	+28.8
I	”	100	5	+20.8

gain with dosage, as shown in Table II.

Over a period of almost 6 weeks, one Rhesus monkey received a total of 3.6 g orally, in doses beginning at 10 mg twice a day, and progressively doubling each week. During this time no digestive or physiological disturbances were observed other than a hematological change. The blood picture altered markedly with the appearance of a decrease in red blood cells and a concomitant decrease in hemoglobin. Fig. 1 describes the rather rapid induction of this anemia, and the almost total recovery during the 39 days after cessation of SN 13592 administration. An attempt to induce the same type of anemia in rabbits and rats was unsuccessful.

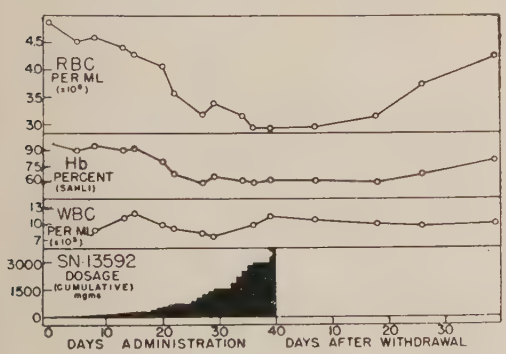

FIG. 1.
Hematological changes upon continued administration of SN 13592 and after its withdrawal.

Local Effects. Ten per cent solutions of calcium pantothenate have been found to have no irritating effect on the conjunctival mucosa of the rabbit.[6] In the present study no gross or microscopic pathology was observed in the vaginal tissues of animals in which SN 13592 was applied to the vaginal mucosa in saline. In 20 treatments over a 10-day period 4 rats received a total of 20 mg each, 2 guinea pigs a total of 50 mg each, and 2 rabbits a total of 200 mg each.

Systemic Effects. The respiratory effect observed upon oral and intravenous administration of SN 13592 led to preliminary studies of its effect on respiration, circulation and smooth musculature. Intravenous administration evoked inconsistent variations in blood pressure as determined by carotid cannulation and a mercury manometer. Doses as high as 50 mg/kg in the cat induced only relatively minor changes in blood pressure. The heart rate showed similar minor variations in this range of drug administration.

The effect of intravenous SN 13592 on respiration is almost immediate, although of

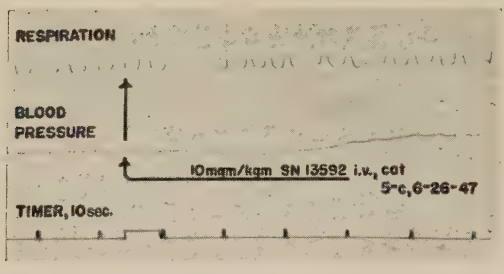

FIG. 2.
Blood pressure and respiratory system effects of SN 13592.

short duration. Respiration became shallower and erratic in the cat after intravenous injection of doses greater than 10 mg/kg (Fig. 2). In some cases it ceased entirely for an interval of a few seconds. Control injections of the vehicle alone failed to produce this response.

It was also found that the compound had a small effect on smooth musculature. *In vitro* intestinal strip preparations were slightly stimulated by 1:2000 dilutions. Ten mg/kg of SN 13592 administered intravenously in the cat enhanced uterine contractility.

Summary. SN 13592 is characterized by an extremely low acute and chronic oral toxicity in several species. Local irritation effects are absent even upon repeated administration. Continued oral feeding of the drug induces an anemia which gradually disappears after the drug is withdrawn. Although there were no consistent effects on blood pressure and heart rate, a respiratory effect was noted. Smooth muscle was generally stimulated by this compound.

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Chemotherapy of Bacteria-Free *Trichomonas vaginalis*. III. Action of Analogues of Pantothenic Acid.*

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The recent demonstration by Kupferberg *et al.*[1] that *Trichomonas vaginalis* requires pantothenic acid motivated this study of the inhibitory effects of pantothenate analogues.

Materials and Methods. The effect of a variety of compounds as growth inhibitors was first explored using strain No. 2 of bacteria-free *Trichomonas vaginalis*.[2] Refined studies were later run using 7 additional pure cultures of *T. vaginalis*. In addition 3 strains of *T. foetus*† and one strain of *T. gallinae* were explored using a single highly active compound.

One ml of an appropriate stock dilution of each compound was added to 9 ml of Simplified Trypticase-Serum medium[1] containing 3.2 μg of added calcium pantothenate per 10 ml of final medium. The solvent used was either water or 95% ethanol. In no case where alcohol was used as a solvent was more than 0.1 ml of alcohol introduced into 10 ml of culture medium. In such instances a control culture containing a like amount of alcohol without drug was carried. The inoculum consisted of 0.05 ml of a 36- to 40-hour culture containing 100,000 trichomonads. The cultures were incubated at 37°C and

* The technical assistance of Mrs. Mary Williams, Miss Ruth Grossman, Mr. Pasquale Russo, and Mr. LeRoy Markle is hereby acknowledged. We wish to thank the staff of the Division of Bacteriology and Serology of the State of New Jersey Department of Health, Trenton, for generous amounts of human blood serum.

1 Kupferberg, A. B., Johnson, G., and Sprince, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 304.

2 Johnson, G., Trussell, M., and John F., *Science*, 1945, **102**, 126.

† The three strains of *T. foetus* used in this study were supplied by Dr. B. B. Morgan, University of Wisconsin.

TABLE I.
Effect of Analogues of Pantothenic Acid upon Strain No. 2 *Trichomonas vaginalis.*

No.		Compound	Observations on 9th day: Lethal at one part in: × 1000	Observations on 9th day: Complete inhibition at one part in: × 1000
1.	SN 13593	(+)-α-γ-Dihydroxy-β,β-dimethyl-N-(2-(phenylsulfinyl)-ethyl)-butyramide	8	16
2.		d(+)-(pantoyltauryl)-p-anisidide	10	20
3.	SN 12610	N-(2-Benzylethyl)-α-γ-dihydroxy-β,β-dimethyl-butyramide. Prepared from natural (—) lactone	400	500
4.	SN 13592	(+)-α-γ-Dihydroxy-β,β-dimethyl-N-(2-(phenylmercapto)-ethyl)-butyramide	750	—
5.	,,	Decolorized by norite	800	—
6.	,,	Crystalline fraction	400	
7.	,,	Fraction boiling up to 55°C at 10–4 mm Hg	400	
8.	,,	Fraction boiling 55-155°C at 10–4 mm Hg	400	
9.	,,	Fraction boiling 155-190°C at 10–4 mm Hg	400	

Compounds 1 and 4 were received from Dr. J. B. Koepfli, California Institute of Technology. No. 2 was supplied by Dr. J. P. English, American Cyanamide Co. No. 3 was supplied by Dr. R. E. Lutz, University of Virginia. Nos. 5, 6, 7, 8, and 9 were prepared by Dr. Wm. Oroshnik and Mr. R. A. Mallory in our own Division of Organic Chemistry.

TABLE II.
Inhibition of Eight Strains of *T. vaginalis* by SN 13592. (Decolorized by Norite.)

Strain No.	Effective dilutions one part in: × 100,000	Not effective at one part in: × 100,000
1	10	11.2
2	8	—
3	14	16
4	7	9
5	9	10
6	6	7
7	2	3
9	9	10

Strain No. 1 was isolated in 1939.[3]
Strains No. 2-No. 7 were isolated in 1945.[2]
Strain No. 9 was isolated in 1947 from a case of acute vaginal trichomoniasis with the assistance of C. E. Folsome, M.D.

observed microscopically after 3, 6, and 9 days.

The data presented in Table I summarize the results obtained with a series of compounds using strain No. 2 as the test organism. Table II shows the comparative sensitivity of 8 strains of *T. vaginalis*.

The data in Table II reveal a sevenfold variation in sensitivity to SN 13592, ranging from 1:200,000 to 1:1,400,000.

The following compounds were found to have little or no activity at a concentration of 1:10,000:

1. dl-N-Pantoylisoamylamine
2. dl-N-Pantoyl-N-butylamine
3. dl-N-Pantoylethanolamine
4. d(+)-Pantoyltauryl-p-nitroanilide
5. d(+)-Pantoyltauryl-p-benzylamide
6. 2-(d(+)-Pantoyltauryl)-amino-5-Chloropyridine
7. d(+)-Pantoyltauryl-p-Carboxyanilide
8. dl-N4-Pantoyltauryl-Sulfanilamide

Compounds 1, 2, and 3 were supplied by Dr. Wm. Shrive, University of Texas. Compounds 4 through 8 were received from Dr. J. P. English, American Cyanamide Company.

Table III shows that the sensitivity of *T. foetus* to the pantothenate analogue is in the same range as that of *T. vaginalis*. The single strain of *T. gallinae* appears to be slightly more sensitive than most strains of *T. vaginalis*. As noted in a previous publication[1] these other trichomonad species grow

TABLE III.
Effect of Norite Treated SN 13592 on *T. foetus*† and *T. gallinae.*

Organism	No. surviving cells at one part in × 10,000
T. foetus 3P	30
,, BR	40
,, AN	50
T. gallinae	100

[3] Trussell, R. E., and Plass, E. D., *Am. J. Obst. and Gynec.*, 1940, **40**, 883.

luxuriantly in the same Simplified Trypticase-Serum medium.

In an effort to determine whether the inhibitory effect of SN 13592 against *T. vaginalis* could be reversed with added Ca-pantothenate, 10 ml of media containing 1:200,000 drug with and without 320 μg of Ca-pantothenate were inoculated. No surviving cells were found in the absence of the Ca-pantothenate. With the added pantothenate the experimental population was equal to that in the control. It is thus suggested that, as with other microörganisms, the analogue of pantothenic acid acts in competition with the natural compound.

Due to its high level of *in vitro* activity it was thought worthwhile to submit SN 13592 to a study of its possible therapeutic activity in infected monkeys. Studies on the pharmacology of the compound by Singher, Millman, and Bosworth[4] made possible a study of the therapeutic effect in cases of human vaginal trichomoniasis.‡

Three monkeys, each weighing 5.5 kg, were successfully infected with a recently isolated strain (No. 9) of *T. vaginalis* which was obtained from a patient with an acute vaginitis.

Monkey No. 1 received 8 doses of 5.5 mg intravenously, followed by 24 oral doses of 300 mg administered twice daily. She remained infected and was therefore treated vaginally. Seven 300 mg doses were administered per vagina. Subsequent microscopic examination of the vaginal fluids revealed a persisting infection.

Monkey No. 2 received 9 oral doses of 275 mg given once daily. The infection persisted in spite of treatment. Seven vaginal doses of 300 mg were then administered without elimination of the infection.

Monkey No. 3 was given a long series of graded oral doses, given twice daily as follows: 10 doses each of 10 mg, 20 mg, 40 mg, and 80 mg, followed by 9 doses of 160 mg, and 2 320-mg doses. The total drug administered amounted to 3.6 g. As in the other 2 monkeys, the infection was not eliminated.

In a series of 6 clinical cases of *Trichomonas vaginalis* vaginitis, a course of vaginal tablets containing 1 mg of SN 13592 was administered twice daily for 2 weeks. All 6 patients retained their infections in the face of the estimated 1:3000 dilution of the drug.

The failure of this analogue of pantothenic acid to eradicate vaginal trichomonad infections is referable to the pantothenic acid content of the blood serum. Denko, Grundy, and Porter[5] demonstrated an average blood concentration of 33 μg per 100 ml. McIlwain and Hawking[6] reported a sensitivity of staphylococci and streptococci to pantoyltaurine. Mice infected with these bacteria were not protected by pantoyltaurine. However, rats which have a lower blood level of pantothenic acid survived such infections when given a protective dose of pantoyltaurine. Woolley and White[7] were unable to produce a pantothenate deficiency in mice by long continued administration of the same compound.

Conclusions. 1. The *in vitro* effectivity of several analogues of pantothenic acid has been demonstrated against *T. vaginalis*. 2. Wide variations in susceptibility of different strains to SN 13592 were found. 3. *T. foetus* and *T. gallinae* were found highly sensitive to this compound. 4. The *in vitro* activity of SN 13592 was reversed by the addition of calcium pantothenate. 5. *In vivo* studies in monkeys and in human beings demonstrated failure of SN 13592 to eradicate the infection.

[4] Singher, H. O., Millman, N., and Bosworth, M. R., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 388.

‡ The authors are indebted to C. E. Folsome, M.D., for these clinical trials.

[5] Denko, W. D., Grundy, W. E., and Porter, J. W., *Arch. Biochem.*, 1947, **13**, 481.

[6] McIlwain, H., and Hawking, F., *Lancet*, 1943, **1**, 459.

[7] Woolley, D. W., and White, A. G. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 106.

16318

Volatile Preservatives for Culture Media.

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During investigations of microbial nutrition centering about growth factors and trace elements, contaminants were repeatedly detected growing in stock solutions of many cp inorganic and organic compounds even when the solutions had been treated with toluene and chloroform, and stored at 6°C. The need for more effective preservatives motivated the study herein reported.

The use of volatile antiseptics is of long standing, especially in work with enzymes. Yet few comparative studies in this field have been described. The common alkyl halide solvents were the compounds tested in some typical studies.[1,2] Propylene oxide and ethylene oxide were recently proposed for sterilizing culture media not to be subjected to heating.[3]

As the solutions to be protected were for use in compounding culture media sterilized by steam, completeness of volatilization of preservative was essential. For the past year, the addition of a mixture of *o*-fluorotoluene, *n*-butyl chloride and 1,2-dichloroethane, has enabled us to store putrescible solutions more or less indefinitely at refrigerator temperatures, and also at room temperature. A description of the development of this preservative may be helpful to others confronted with a similar problem, and may indicate some interesting possibilities in the field of volatile antiseptics.

Materials and Methods. a. *Compounds.* The following considerations guided selection of compounds to be tested:

1. Boiling point $< 121°C$.

2. Immiscibility with water to ensure removal by steam distillation on autoclaving.

3. Satisfactory chemical stability in contact with water at room and sterilization temperature.

4. Low reactivity towards most compounds used in culture media. Where some decomposition might occur, the products to be nutritionally inert.

5. Moderate cost and ready availability.

At the beginning of this investigation a specific gravity near 1.0, to allow a more stable dispersion in dilute aqueous media, was considered a desideratum, but later experiments indicated advantages in blends of light and heavy compounds, and hence less importance was attached to this property. Compounds boiling at or below room temperature were too easily lost from opened containers. Hydrocarbons were excluded because they were too easily metabolized by microorganisms.[4] Nevertheless because the use of toluene was traditional in enzyme studies, some preliminary experiments with it were conducted. It proved very inefficient: toluene-saturated broth allowed growth of *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Streptococcus faecalis,* and *Torula utilis;* however it did check more or less completely *Bacillus cereus* and *Mycobacterium lacticola.* It was decided to concentrate attention on alkyl and aryl halides because these appeared best to fulfill the above listed requirements and they already had shown some effectiveness. Few tests were conducted with compounds with allylic halogen atoms because of their instability. Some sulfur-containing compounds were investigated despite their instability and unpleasant odors. Nearly all compounds tested were redistilled in an all-glass apparatus. "Practical" grade chemicals were distilled over a 3° range; nearly all other chemicals boiled over a much narrower range, agreeing well with the constants in the litera-

1 Joachimoglu, G., *Biochem. Z.*, 1921, **124**, 130.
2 Libnell, D., *Arch. Mikrobiol.*, 1935, **6**, 326.
3 Hansen, H. N., and Snyder, W. C., *Phytopath.*, 1947, **37**, 369.
4 Zobell, C. E., *Bact. Revs.*, 1946, **10**, 1.

TABLE I.
Compounds Tested.

Toluene	1-bromo-2-methyl-propane (*iso*-butyl bromide)
Diethyl ether	4-bromo-2-methyl butane (*iso*-amyl bromide)
Iso-propyl ether	1,2-dibromoethylene
Carbon tetrachloride	1,1-dibromoethane (ethylidene bromide)
Chloroform	1-bromo-1-propene
Dichloromethane (methylene chloride)	2-bromo-1-chloro-propane
1-chloropropane (*n*-propyl chloride)	chlorobromomethane
2-chloropropane (*iso*-propyl chloride)	1,2-chlorobromoethane (1,2-ethylene chlorobromide)
1-chlorobutane (*n*-butyl chloride)	Iodoethane (ethyl iodide)
Monochlorbutenes	1-iodo-propane (*n*-propyl iodide)
2-chloro-2-methyl-1-propane (*t*.-butyl-chloride)	2-iodo-butane (*sec*.-butyl iodide)
4-chloro-2-methyl butane (iso-amyl chloride)	*p*-fluorotoluene
1-chlorohexane (*n*-hexyl chloride)	*o*-fluorotoluene
1,1-dichloroethane (ethylidene chloride)	Fluorobenzene
1,2-dichloroethane (ethyiene chloride)	Benzotrifluoride
1,1,1-trichloroethane (methyl chloroform)	1,1,2-trichloro-3-trifluoro-1-propene
1,2-dichloroethylene	Thiophene
1,1,2,2-tetrachlorethylene (carbon dichloride)	2-methyl thiophene
1-chloro-1-propene	3-methyl thiophene
Bromoethane (ethyl bromide)	Dimethyl disulfide
1-bromo-1-butane (*n*-butyl bromide)	Methyl ethyl sulfide
2-bromo-2-methyl propane (*t*.-butyl bromide)	Methyl orthoacetate

TABLE II.
Test Organisms

Routine test organisms	Source	
Escherichia coli	R. R. Roepke	ATCC* 9723
Protaminobacter alboflavum b	C. B. van Niel	
Pseudomonas riboflavina	J. W. Foster (available from ATCC)	
Corynebacterium pseudodiphthericum		ATCC 6981
Staphylococcus aureus	Merck and Co., Inc.	" 9144
Streptococcus faecalis		" 8043
Actinomyces scabies	W. H. Burkholder	" 3021
Bacillus subtilis		" 6633
Torula utilis		" 8206

* ATCC—American Type Culture Collection, Georgetown University School of Medicine, Washington, D.C.

ture and commercial catalogs. The compounds tested are listed in Table I. For the sake of brevity, source and physical constants are omitted. Most of the chemicals were obtained either from the Eastman Kodak Co., the Amend Drug and Chemical Co., or the Columbia Organic Chemical Co., Inc., Columbia, S.C. The methyl chloroform and thiophenes were gifts of the Dow Chemical Co. and the Socony-Vacuum Laboratories respectively. Where alternate names are in use, the one employed by *Chemical Abstracts* is given first.

b. *Test Organisms.* The organisms were selected to represent some of the genera commonly encountered as air contaminants in the ordinary course of laboratory work. Mycelial fungi were not included because they never appeared in preservative-treated solutions. It was supposed that acid-fast bacteria might be important contaminants, but they were not encountered, and hence a petroleum-utilizing strain of *Mycobacterium lacticola* and a rapidly-growing strain of *M. avium* were dropped from the roster of routine test organisms. They were killed by nearly all the halogenated compounds tried. The organisms selected for routine use are listed in Table II. Additional organisms were used in later tests.

c. *Test Procedure.* Ten ml amounts of broth, whose composition is given in Table III, were distributed in 125 x 20 screw-capped tubes and sterilized for 20 minutes at 117 to 121°C. The medium allowed heavy rapid growth at room temperature of all the routine test organisms. The tubes were inoculated with a drop of fresh slant growth suspended in broth. After 4 hours at room temperature,

TABLE III.
Composition of Test Medium.

	%
K_2HPO_4	0.05
$MgSO_4 \cdot 7H_2O$	0.01
Na_3 citrate $\cdot 2H_2O$	0.1
Trypticase*	0.6
Lactate	0.25
Glycerol	0.5
Solubilized Liver†	0.1
pH 6.9 to 7.1	

* Baltimore Biological Laboratory.
† Wilson's Liver "L."

the compounds to be tested were aseptically pipetted in, to a final concentration by volume of 1%. The tubes were shaken vigorously and then allowed to stand at room temperature (21 to 28°C) for 96 to 120 hours. Growth was then recorded. To test for completeness of removal of the preservative on autoclaving, and absence in the medium of toxic residual decomposition products, the materials under test were added to broth, which was then autoclaved according to the routine technic. With this method, after sterilization, the exhaust valve of the autoclave was kept shut until the partial vacuum which develops on cooling was subsequently dissipated by the gradual influx of air. If necessary the exhaust valve was opened very slightly. This procedure was equivalent to steam distillation under reduced pressure and no difficulty was experienced in complete removal of preservatives, even in amounts well over the 1% level. To illustrate the effectiveness of the method, trials with *n*-hexyl chloride (b.p. 132.6°C) showed it completely removed under the standard conditions. In orientation experiments it was convenient to test for non-volatilized preservative by adding a few drops of I-KI solution; free iodine dissolving in the preservative made tiny droplets conspicuous.

Mixtures were compounded on a volume basis. In practice, a few drops of preservative were added to the solution in a glass-stoppered bottle and the bottle was then shaken vigorously. Volumes of culture media up to 2 liters were thus successfully preserved for months in the refrigerator.

Results. After conducting killing tests in this manner on 16 compounds, the majority of them halogenated hydrocarbons, it ap-

TABLE IV.
Killing Tests with Mixtures of *o*-Fluorotoluene, *n*-Butyl Chloride, and 1,2-Dichloroethane.

	None			Routine No. 3*	*o*-Fluoro-toluene	*n*-Butyl chloride	1,2-dichloro-ethane	*o*-Fluorotoluene—*n*-Butyl chloride—1,2-dichloroethane			
								1:1:1	1:2:1	1:3:1	1:1:2
Escherichia coli	+	++	++	0	0	++	+++	0	0	0	0
Protaminobacter alboflavum b	+++	+++	+++	0	0	++	+++	0	0	0	0
Pseudomonas riboflavina	+++	+++	+++	0	0	+++	+++	0	0	0	0
Corynebacterium pseudodiphthericum	+++	+++	+++	0	0	+++	0	0	0	0	0
Staphylococcus aureus	++	+++	+++	0	++	++	++	0	0	0	0
Streptococcus faecalis	++	+++	+++	0	++	+++	++	0	0	0	0
Actinomyces scabies	+	+++	+++	0	0	+++	0	0	0	0	0
Bacillus subtilis	++	+++	+++	0	++	+++	++	0	0	0	0
Torula utilis	++	+++	+++	0	++	+++	++	0	0	0	0

* Routine No. 3
33% 1-Bromo-1-propene
67% *n*-Butyl chloride

0 = growth
+ = growth

peared that a 3:1 mixture of *n*-butyl chloride and CCl_4 might be satisfactory. Need for a better preservative later became evident, since contaminations of stock solutions, while much less frequent, still appeared; and under test conditions there was some growth of *E. coli, Staphylococcus aureus, Streptococcus faecalis* and *Torula utilis*. The next mixture extensively used consisted of equal parts of CCl_4, *n*-butyl chloride, and 1,2-dichloroethane. This was much more effective but did not completely check the growth of *E. coli* and *Strep. faecalis*. Complete effectiveness was shown by a mixture of 1 part 1-bromo-1-propene and 2 parts *n*-butyl chloride. This not only completely inhibited the routine test organisms and many others, but no contaminants appeared in practice; 1-bromo-1-propene was not altogether stable, yellowing slowly at room temperature. This did not significantly alter its antiseptic properties, and volatility tests with yellowed samples remained satisfactory. However on continued use it became extremely irritating to the eyes and nose, and had to be discarded. Several of the somewhat less effective halides were tried singly, and many were tried in combination with *n*-butyl chloride and 1,2-dichlorethane. *O*- and *p*-fluorotoluene appeared unusually effective in repeated trials and were then tested in detail in combination with other halides. The *o*-isomer appeared somewhat more effective and is part of the preservative now in use. A typical experiment with the present preservative is shown in Table IV. About 50 experiments in all were performed on this scale.

The sulfur compounds were almost completely non-inhibitory. No consistent differences emerged between bromo and chloro homologues. The iodo derivatives were not effective enough and were too unstable to encourage further work in that direction.

The preservative finally adopted consisted of a mixture of 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1,2-dichlorethane.

Discussion. Rhodotorulae had previously been isolated as principal contaminants of solutions preserved with the less effective alkyl halide-containing mixtures. Their abundant growth in such seemingly unfavorable media as 3% boric acid has been described.[5] They have not appeared in any of the solutions preserved with the new mixture. Exposure of varied agar plate media indicated that rhodotorulae were quantitatively insignificant but constantly present in our laboratory air. As they have been reported to be pyrogenic,[6] they may perhaps constitute an important source of pyrogenic contamination of therapeutic preparations.

Certain limitations attend the use of alkyl and aryl halide preservatives. Compounds of this class react slowly with –SH groups.[7] This does not appear to affect the practical usefulness of these compounds in preservation of natural materials such as yeast extract and protein hydrolysates, probably because most of the potential –SH compounds are in the disulfide form. This reaction became evident when concentrated solutions of Na thiosulfate were treated with the preservative: the solutions became toxic and acquired peculiar odors. Slow interaction with –SH compounds may be of significance in accounting for the effectiveness of the preservative. Little more can be said at present as to their mode of action. In the recommended preservative mixtures, the most effective component is *o*-fluorotoluene. The *n*-butyl chloride, lighter than water, is a poor preservative but serves to bring the specific gravity of the mixture closer to that of water, besides being an inexpensive extender for the relatively expensive *o*-fluorotoluene. The 1,2-dichloroethane may function as a contact agent because of its relatively good solubility in water (*ca.* 0.9% at room temperature) bringing the less soluble *o*-fluorotoluene into more effective contact with the cell. The obvious fat-solvent properties of these compounds appear inadequate to account for their striking differences in effectiveness.

The tremendous recent expansion in the

[5] Skinner, C. E., *et al.*, 2nd ed. *Henrici's Molds, Yeasts, and Actinomycetes*, 1947, John Wiley and Sons, N.Y.

[6] Co Tui, F. W., and Schrift, M. H., *J. Lab. Clin. Med.*, 1942, **27**, 569.

[7] Hickinbottom, W. J., *Reactions of Organic Compounds*, p. 106, Longmans, Green and Co., 1936, New York.

laboratory and industrial chemistry of organic fluorine compounds[8] may, in the light of the results reported here, encourage further work in the rather neglected field of the water-immiscible volatile antiseptics.

8 Symposium on Organo-fluorine Chemistry, 1947, *Ind. Eng. Chem.* (many authors), **39**, 235.

Summary. A mixture by volume of 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1,2-dichlorethane, is recommended as a chemically nearly inert but nevertheless effective preservative for biological solutions. This preservative, used at approximately the 1% level, is completely removed under the usual conditions of steam sterilization.

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Isolation of an Agent in Chicken Embryo Causing Infectious Sinusitis of Turkeys.

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The term infectious sinusitis of turkeys was suggested by Dickenson and Hinshaw[1] to differentiate it from sinusitis which may occur in connection with reduced vitamin A intake[2] or follow inoculation with *Hemophilus gallinarum*.[3] Infectious sinusitis occurs in all sections of the country and although mortality is low, morbidity varies from 10% to 90% of a flock.[1,4] Thus, losses to the grower resulting from inferior quality of the birds are often quite heavy. According to the literature Delaplane[5] has reported a *Pasteurella*-like organism to be associated with an infectious type of sinusitis. In transmission experiments Hart[6] and Hinshaw[7] were unable to filter the causal agent through collodion membranes, Chamberland or sintered glass filters.

Serial transmission of the disease in this laboratory from a case of sinusitis submitted to the laboratory for examination was carried out in the usual manner.[7] Sinus exudate was aspirated from infected turkeys and 0.5 ml amounts were inoculated into the right infraorbital sinus of normal turkeys. The inoculated sinus became swollen 5 to 10 days after inoculation and contained a viscous exudate which resembled egg albumen and was bacteriologically sterile. Later the sinuses became greatly distended and often contained caseated material from which a variety of bacteria were isolated. On the assumption that these organisms represented secondary bacterial invaders an attempt was made to isolate the causal agent early in the course of the disease from the third serial turkey passage. Accordingly, sinus exudate was aspirated on the second day after the appearance of clinical symptoms and was inoculated into embryonating eggs by the following routes: onto the chorioallantois, into the allantoic cavity and into the yolk sac. No embryo deaths or significant macroscopic lesions were observed in any of the eggs inoculated onto the chorioallantois or into the allantoic cavity. However, all of the 10 embryos inoculated into the yolk sac died 6 to 12 days after inoculation. Yolk sacs from these embryos were bacteriologically sterile and were subsequently passed in

1 Dickenson, E. M., and Hinshaw, W. R., *J. Am. Vet. Med. Assn.*, 1938, **93**, 151.

2 Hinshaw, W. R., and Lloyd, W. E., *Hilgardia*, 1934, **8**, 281.

3 Beach, J. R., and Schalm, O. W., *Poult. Sc.*, 1936, **15**, 466.

4 Lee, C. D., *No. Am. Vet.*, 1942, **23**, 715.

5 Delaplane, J. P., *Poult. Sc.*, 1944, **23**, 247.

6 Hart, L., *Austral. Vet. J.*, 1940, **16**, 163.

7 Hinshaw, W. R., from *Diseases of Poultry*, edited by H. E. Biester and L. Devries, The Iowa State College Press, 1945.

chicken embryos by yolk sac inoculation for a total of 28 consecutive passages.

After the second yolk sac passage 0.5 ml of a 10% yolk sac suspension was inoculated into the right sinus of each of 2 normal turkeys. The sinuses became swollen 5 and 8 days after inoculation respectively. Sinus exudate aspirated on the first day after the appearance of clinical symptoms was bacteriologically sterile and killed all of 10 chicken embryos 6 to 12 days after inoculation into the yolk sac. Clinical sinusitis was produced in a total of 10 additional turkeys with yolk sac material from the 3rd, 4th, and 16th egg passages respectively.

Filtration studies on the egg-adapted strain of the agent of turkey sinusitis were carried out using a 1/100 dilution of infected yolk sacs. At least 50.0 ml amounts of the suspension were filtered through Berkefeld V, Berkefeld N, and Seitz E K filters respectively. The filtrates obtained, together with the unfiltered suspension, were tested for infectivity by inoculation into groups of 15 embryonating eggs. All embryos inoculated with the unfiltered suspension and the Berkefeld V filtrate died 4 to 8 days after inoculation. However, all embryos inoculated with Berkefeld N and Seitz E K filtrates respectively were still living 14 days after inoculation. It is evident that the causal agent passed through a Berkefeld V filter but did not pass readily Berkefeld N or Seitz E K filters.

During the first 10 egg passages microscopic examination of impression smears of infected yolk sacs stained by Machiavello's technic revealed only an occasional red staining coccoid body. However, beginning with the 14th egg passage when the infective titer of yolk sacs reached 10^{-8} or more small, coccoid bodies were regularly observed both intra- and extracellularly. These bodies were found to vary in size from approximately 0.5 μ to 1.0 μ or more in diameter and were frequently imbedded in a matrix. The bodies described above closely resembled morphologically the bodies found in similar impression smears of yolk sacs infected with the etiological agents of the lymphogranuloma-psittacosis group (*Chlamydozoaceae*).

Further studies on the characteristics of the agent of turkey sinusitis and its possible etiological relationship to an agent isolated from air sac infection in turkeys by Minard and Jungherr[8] are in progress.

Summary. 1. An agent capable of producing clinical sinusitis in turkeys was isolated and propagated in the yolk sac of the developing chicken embryo. 2. Impression smears of infected yolk sacs stained by Machiavello's technic revealed morphologic forms closely resembling those found in the lymphogranuloma-psittacosis group of agents (*Chlamydozoaceae*).

8 Minard, E. L., and Jungherr, E., 1944, Proc. 16th An. Conf. Lab. Workers in Pullorum Dis. Control, Coll. of Agr., Univ. of Conn., mimeog. report.

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Observations on the Effect of 4-Amino-Pteroylglutamic Acid on Mice.

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The production of a syndrome in mice by feeding a crude "antagonist" of pteroylglutamic acid (PGA) and its prevention by raising the dietary level of PGA was described.[1] The syndrome was marked by slow growth followed by a loss in weight, low hemoglobin and low white blood cell count. Recently, another substance, "4-amino" pteroylglutamic acid ("4-amino-PGA"), which is strongly

1 Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 368.

antagonistic to PGA in the *Streptococcus fecalis* R test, was described.[2] This compound differs from PGA only in having an amino group in place of the hydroxyl group on the pteridine ring. Its inhibition of the growth of *S. fecalis* R was reversed by increasing the level of PGA. The effect of the compound in the nutrition of mice was studied in the present investigation.

Experimental. The animals received a purified diet without PGA and with 1% succinylsulfathiazole, identical with the diet previously described[1] except that *p*-aminobenzoic acid was omitted in Experiment 2. In a preliminary experiment it was found that mice died within a few days after being placed on a diet containing levels of 1 mg of 4-amino-PGA or more per kilo. Accordingly in Experiment 1 low levels of 4-amino-PGA were fed with various amounts of PGA ranging up to comparatively high levels. The results are in Table I. At a level of 0.3 mg the 4-amino compound had a slight tendency to depress the blood values which was reversed by high levels of PGA. When the level of the compound was raised to 1.0 mg, mortality was high even with large amounts of PGA.

In Experiment 2, the effects of PGA upon mice receiving 4-amino-PGA was explored further. Higher levels of 4-amino-PGA were used, ranging up to 3 mg per kilo of diet. Some reversal of the toxic effect was observed at the 0.3 mg level, but no protection was afforded by PGA when the 4-amino compound was fed at levels of 1 or 3 mg per kilo of diet (Table II).

No lesions were visible upon gross examination at autopsy.

Summary. The 4-amino analogue of pteroylglutamic acid was fed to mice as a dietary supplement. There was some indication of reversal by pteroylglutamic acid of the effect on mice when the level of the antagonist was 0.3 part per million of diet. Death was found to occur within a few days with levels of 1 part per million of diet or higher. The effect was not reversed by feeding high levels

TABLE I.
Effects of Various Levels of PGA and 4-Amino PGA on Growth and Hematology of Mice. Six Mice Were Used in Each Group.

Group	Supplement in mg per kilo of diet		Body wt and No. of survivors					Hemoglobin g per 100 cc		White cells ($\times 10^3$) per mm^3	
	PGA	4-amino PGA	0	1	2	4	5 wk	at 2 wk	at 4 wk	at 2 wk	at 4 wk
1	0.1	0	11	16	17	21 (5)	21 (5)	10.3	13.0	7.1	10.0
2	1.0	0	11	16	17	21	22 (6)	14.3	14.4	12.5	9.4
3	10	0	11	16	18	21	22 (6)	16.2	17.6	8.8	11.8
4	100	0	12	18	19	23	23 (6)	13.7	16.4	9.0	13.0
5	0.1	0.1	13	17 (5)	18	20	19 (5)	11.3	12.2	11.4	7.3
6	1.0	0.1	12	18	21	26	26 (6)	15.8	15.8	11.8	13.4
7	10	0.1	12	18	21	25 (5)	24 (4)	13.4	15.8	13.2	11.8
8	100	0.1	12	17	19	23	24 (6)	15.6	16.9	12.8	10.9
9	0.1	0.3	13	16	15 (5)	20	20 (5)	8.5	10.4	7.4	5.6
10	1.0	0.3	12	14 (5)	16	20	21 (5)	13.9	13.4	15.3	9.1
11	10	0.3	12	19	22	24	24 (6)	14.1	15.7	14.4	11.9
12	100	0.3	13	18	20	22	21 (6)	15.7	17.2	14.5	16.8
13	10	1.0	13	12 (3)	14 (1)	(0)	(0)	12.1	—	10.3	—
14	100	1.0	11	14 (2)	17 (2)	22 (2)	23 (2)	10.9	11.5	7.0	10.2

[2] Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

TABLE II.
Effects of PGA and 4-Amino PGA on Growth and Survival of Mice in Experiment 2. Five mice were used per group.

Group	Supplement in mg per kg of diet		Body wt and (in parentheses) No. of survivors					Avg survival time in days*
	PGA	4-amino PGA	0	1	3	5	8 wk	
15	0.1	0	10	14	21	24	22 (5)	
16	1.0	0	10	13	19	19	19 (5)	
17	10	0	10	15	20	22	24 (5)	
18	100	0	11	13	20	22	24 (5)	
19	0.1	0.1	10	15	20	24	24 (5)	
20	1.0	0.1	10	16	21	26	28 (5)	
21	10	0.1	10	16	21	23	24 (5)	
22	100	0.1	11	16	23	26	30 (5)	
23	0.1	0.3	10	(0)				4
24	1.0	0.3	10	13 (4)	20	23	26 (4)	
25	10	0.3	10	13	18	20	21 (5)	
26	100	0.3	9	14	18 (4)	21	24 (4)	
27	0.1	1.0	10	9 (1)	(0)			5
28	1.0	1.0	10	13 (1)	(0)			8
29	10	1.0	10	9 (1)	(0)			7
30	100	1.0	10	(0)				5
31	0.1	3.0	10	(0)				6
32	1.0	3.0	11	(0)				4
33	10	3.0	10	(0)				4
34	100	3.0	10	(0)				4

* In the case of groups in which all the animals died during the experimental period.

of pteroylglutamic acid. This finding may be contrasted with the inhibitory effect of the 4-amino compound on *S. fecalis* R which is reversed by pteroylglutamic acid.

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Relation Between Induced Hyperthyroidism and an Unidentified Chick Growth Factor.*

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The use of biological assays for study of unidentified growth factors is often restricted by the extent to which they can measure low concentration of a factor in a given material. This may be due to the small response of the animal to a given supplement. A series of studies have shown that the chick requires an unidentified growth factor found in condensed fish solubles,[1] whole liver powder, and certain other liver fractions.[2,3] The increased growth rate of the chick as a result of adding supplements carrying the factor has been used as an assay in determining some of the prop-

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1 Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Science,* 1948, in press.

2 Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *Poultry Science,* 1948, in press.

3 Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.,* 1947, **170**, 419.

TABLE I.
Effect of Supplements on the Growth Rate of Chicks.

Trial No.	Lot No.	Supplements to basal ration	Avg wt at 4 wks	Mortality %
1	1	None	120	25
	2	3% condensed fish solubles	220	0
	3	.25% desiccated thyroid U.S.P.	129	42
	4	.25% " " + 3% condensed fish solubles	203	25
	5	.25% " " + .05 cc Reticulogen (Lilly)/bird/day*	171	25
2	6	None	178	0
	7	3% condensed fish solubles	249	0
	8	.125% desiccated thyroid	137	0
	9	.125% " " + 3% condensed fish solubles	279	0
3	10	None	141	17
	11	3% condensed fish solubles	205	0
	12	.03% iodinated casein	96	33
	13	.03% " " + 3% condensed fish solubles	238	8
4	14	None	129	15
	15	3% condensed fish solubles	191	15
	16	.125% desiccated thyroid	134	40
	17	.125% " " + 3% condensed fish solubles	221	0
	18	.02% iodinated casein	117	40
	19	.02% " " + 3% condensed fish solubles	232	5
5	20	None	159	0
	21	3% condensed fish solubles	259	0
	22	.05 cc Reticulogen/bird/day*	240	0
	23	.125% desiccated thyroid	156	17
	24	.125% " " + 3% condensed fish solubles	249	17
	25	.125% " " + .05 cc Reticulogen/ bird/day*	233	0
	26	.02% iodinated casein	122	8
	27	.02% " " + 3% condensed fish solubles	273	0
	28	.02% " " + .05 cc Reticulogen/ bird/day*	251	0

* Injected.

erties of the factor.[4] However, in more recent work (unpublished) with low concentrations of the factor the response obtained is sometimes such that it is questionable whether it may be attributed to the presence of the factor, or merely to normal variation between the experimental groups of chicks.

The recent work of Betheil, Wiebelhaus, and Lardy[5] and of Ershoff *et al.*[6] offers a method which may be helpful in improving certain biological assays for unidentified growth factors. They found that the administration of desiccated thyroid to rats resulted in poor growth and a shortened survival period. It was suggested that feeding desiccated thyroid increases the requirement of the growing animal for some unknown factor(s), and that this increased requirement is fulfilled by feeding materials containing the unidentified antithyrotoxic factor. Liver and yeast were found to be sources of the antithyrotoxic material. It has been long recognized that hyperthyroidism increases the animals' requirement for various nutrients. The relationship of experimentally induced hyperthyroidism to increased requirements of many of the known vitamins has been reviewed.[5]

The observations of Betheil *et al.*[5] suggested that production of a condition of hyperthyroidism in chicks might be useful in

4 Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Biol. Chem.*, 1948, **173**, 117.

5 Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

6 Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 500.

increasing the effective assay range, and sensitivity of chicks to the unidentified chick growth factor found in condensed fish solubles and certain liver fractions. The present investigations were initiated to study the effect of thyroid active materials on the degree of response of chicks to supplements containing the unidentified factor or factors.

Experimental. Day-old (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) chicks were used in all the studies. The chicks were progeny of hens fed diet B1, described in a previous report.[1] The experimental groups were housed in electrically heated batteries with raised screen floors. The chicks were wing-banded and weighed at the beginning of the experiment and at weekly intervals thereafter. Feed and water were supplied *ad libitum*. The experiments were terminated at the end of four weeks. The basal diet used was the same as that used in previous trials.[1] It consisted of the following ingredients: ground yellow corn 35, wheat bran 10, wheat middlings 10, dehydrated alfalfa meal 5, soybean oil meal 28, vitamin test casein 7.5, limestone grit 2.0, steamed bonemeal 1.5, iodized salt 0.5, fish oil (2000A-400D) 0.5, $MnSO_4H_2O$ 0.025 g; thiamine 0.3, riboflavin 0.6, niacin 5.0, calcium pantothenate 2.0, pyridoxine HCl 0.4, inositol 100, choline chloride 150, *p*-aminobenzoic acid 10, biotin 0.02, folic acid 0.05, menadione 0.05, α-tocopherol 0.3 mg. Supplements to the basal ration were made at the expense of the corn.

Results. The results obtained are presented in Table I. In trial 1 the addition of 0.25% desiccated thyroid to the basal diet resulted in slow growth and high mortality (lot 3). The growth rate in this case was not significantly different from that of the basal group (lot 1). The addition of either 3% condensed fish solubles to the diet containing desiccated thyroid; or the daily injection of 1 U.S.P. unit of reticulogen (Lilly), a concentrated liver extract used in the treatment of pernicious anemia, gave a significant increase in growth rate. In both cases, however, the average weight attained at 4 weeks of age was somewhat below that of the positive control group in which condensed fish solubles alone was added to the basal ration (lot 2).

In the second trial a lower level of desiccated thyroid was used. In this case, a level of .125% desiccated thyroid in the diet (lot 8) resulted in a significantly lower growth rate than was obtained with the basal group (lot 6). The addition of 3% condensed fish solubles and .125% desiccated thyroid to the basal diet (lot 9) resulted in a significant increase in growth above that of the positive control (lot 7) which was supplemented with fish solubles alone.

Another source of thyroidal activity was tested in trial 3. Iodinated casein (3.07% thyroxin) was added to the basal ration at a level of .03%. A decrease in growth rate and an increase in mortality resulted (lot 12). The effect obtained when 0.03% iodinated casein and 3% condensed fish solubles were added was similar to that observed in the previous trial using desiccated thyroid. The average weight at 4 weeks of age (lot 13) was significantly above that of the positive control (lot 11).

The results in trial 4 are essentially in agreement with those of the previous 2 trials. The addition of desiccated thyroid to the basal ration (lot 16) did not result in a lower rate of growth than was obtained with the basal ration alone, but was accompanied with a marked increase in mortality. Iodinated casein (lot 18) caused both a decreased growth rate and high mortality. When the basal ration was supplemented with either of these materials along with 3% condensed fish solubles (lots 17, 19) a growth response greater than that which was obtained with condensed fish solubles alone (lot 15) resulted.

Trial 5 was carried out to determine whether or not reticulogen injections would fully substitute for condensed fish solubles in rations containing iodinated casein or .125% desiccated thyroid. The results show that reticulogen was effective in both cases (lots 25, 28) in overcoming the thyrotoxic effect of these materials. The administration of desiccated thyroid did not cause a decreased growth rate (lot 23). The growth rate was depressed in the group fed 0.2% iodinated casein (lot 26). In both cases mortality was higher than in the control groups. The addition of con-

densed fish solubles and iodinated casein gave an added growth response, but this effect was not observed with desiccated thyroid and fish solubles.

Discussion. The data presented here show that either condensed fish solubles or reticulogen (Lilly) serve to counteract the thyrotoxic effect of desiccated thyroid in the chick. This result parallels the observations of Ershoff *et al.*[6] and of Betheil *et al.*[5] who noted the anti-thyrotoxic effect of liver and yeast when desiccated thyroid was fed to rats.

A similar protective action was also obtained when iodinated casein was used in place of desiccated thyroid. This would indicate that the thyrotoxic effect is attributable to an increased thyroid activity. Since the protective action is exerted by both condensed fish solubles and reticulogen (Lilly) it would appear that the anti-thyrotoxic factor is identical to the unidentified growth factor described previously.[1,3]

In addition to the protective action of condensed fish solubles a further effect was noted in these experiments. An increased growth rate was obtained when either 0.125% desiccated thyroid or .02-.03% iodinated casein was added to the ration containing condensed fish solubles. The effect is similar to that observed by Irwin, Reinecke and Turner[7] who reported a stimulation in growth of chicks when iodinated casein was added to the ration. However, in the experiments reported herein the addition of either desiccated thyroid or iodinated casein to the basal ration without fish solubles caused a decreased growth rate and high mortality and it was only in the presence of an adequate amount of the factor supplied by condensed fish solubles that a growth stimulation was obtained.

The levels of iodinated casein that were found to be effective were considerably lower than those employed by Irwin *et al.*[7] They reported that 36 g of iodo-casein (3.1% thyroxin) per cwt. of feed was the optimum dosage level and that variations from this level were not effective. In the experiments reported herein the effective levels ranged from 9.08 to 13.62 g iodinated casein (3.07% thyroxin) per 100 lb. of feed. Higher levels were not tested. In a recent report Wheeler, Hoffman, and Graham[8] found that a diet containing 10 g of thyroprotein (3.0% thyroxin) per cwt. of feed resulted in a significant increase in uniformity and mean body weight of male birds at 12 weeks of age. The level of iodinated casein used was within the range we found to be effective.

The growth stimulation obtained may be due to an increase in the basal metabolic rate of the chick. Evidence that the administration of thyro-active materials to animals causes an increase in the requirement of many nutrients has been adequately reviewed by Betheil *et al.*[5] It appears that in the presence of sufficient amounts of all the nutrients, the increased basal metabolic rate results in a more rapid growth rate than is exhibited by the normal or untreated individual rather than a thyro-toxicity. It is quite evident, however, that a deficiency in the ration of the unidentified factor(s) found in condensed fish solubles results in a thyro-toxicity when desiccated thyroid or iodinated casein is administered.

The results that have been obtained by various investigators would seem to be dependent on 2 factors: the completeness of the ration and the level of thyro-active material fed. The ration used by Wheeler *et al.*[8] containing both fish meal and meat meal as protein supplements, would likely meet both of these requirements; however the basal ration used by Turner, Irwin, and Reinecke[9] and by Irwin *et al.*[7] might be submarginal with regard to the unidentified factor of condensed fish solubles.

The results of the study indicate that an experimentally induced hyperthyroidism may be an extremely useful tool in biological assay for unidentified factor(s) required by the chick for optimum growth. A level in the diet of .125% desiccated thyroid or levels of .02 to .03% iodinated casein resulted in an

7 Irwin, M. R., Reineke, E. P., and Turner, C. W., *Poultry Science*, 1943, **22**, 374.

8 Wheeler, R. S., Hoffman, E., and Graham, C. L., *Poultry Science*, 1948, **27**, 103.

9 Turner, C. A., Irwin, M. R., and Reinecke, E. P., *Poultry Science*, 1944, **23**, 242.

increase in the assay range for the unidentified factor.

The increase in growth rate which was obtained when iodinated casein or desiccated thyroid was added to the ration containing condensed fish solubles has not been studied beyond 4 weeks of age. Work is now in progress to determine the possible value of a combination of fish solubles and iodinated casein in commercial feeding.

Summary. A thyrotoxic condition in the chick, induced by feeding desiccated thyroid or iodinated casein was effectively counteracted either by supplementing the diet with condensed fish solubles or by the injection of reticulogen.

An increased growth response was obtained upon the addition of either desiccated thyroid or iodinated casein to a ration containing adequate amounts of the known and unidentified chick growth factors.

The addition of either 0.125% desiccated thyroid or 0.02 to .03% iodinated casein to the basal ration resulted in an improved assay range for the unidentified chick growth factor(s) in condensed fish solubles and reticulogen.

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